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ANTIBODY PRODUCTION BY SINGLE CELLS.
III. THE HISTOLOGY OF ANTIBODY PRODUCTION

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FOLLOWING the subcutaneous injection of an antigen, the lymph node draining the injection site undergoes an interesting series of histological changes. The final result of these changes is the appearance of plasma cells. The draining lymph node is an important site of antibody production, and in previous experiments of this series we have studied antibody production by single cells removed from such nodes and incubated in microdroplets (Nossal and Lederberg, 1958; Nossal, 1959*b*). The technique used depended on the immobilization of motile Salmonellae by antiflagellar antibody. In those experiments, the cells studied were selected at random from a heterogeneous population. In the present experiments, advantage was taken of the fact that the different cells present can be identified in the living, unstained state with about 90 per cent accuracy. It proved possible to test representatives of all the different cell types for antibody production. It was found that almost all the antibody-producing cells belonged to the plasma cell series.

MATERIALS AND METHODS

Animals.—Adult male Wistar albino rats were used. They were fed on a diet of mouse pellets and tap water *ad libitum*.

Bacteria.—The monophasic organism *Salmonella adelaide*, flagellar antigens H_1^{fg} was used. Bacteria were maintained at maximal motility by frequent passages through a semi-solid nutrient gelatin agar medium (Lederberg, 1956). Fresh four-hour broth cultures were used for motility inhibition tests.

Antigen.—The antigen used was a preparation of isolated flagella from *Salm. adelaide* (Nossal, 1959*a*). About 0·5 ml. of flagellar material was dissolved in 10 ml. of sterile physiological saline and termed flagellar antigen. This antigen was sterile on culture in nutrient broth and nutrient agar.

Immunization of animals.—Rats were immunized by injecting 0·25 ml. of 1 : 100 dilution of flagellar antigen in physiological saline into each hind foot-pad. Animals used for study of the primary response were killed 3–12 days after immunization. Animals used for the study of the secondary response were given a second pair of similar injections 4 weeks after the primary injections, and killed 1–12 days later. Control studies were done on normal rats, and on one rat immunized by several food-pad injections of sheep red blood cells.

Preparation of cell suspensions and micromanipulation.—The techniques used for the preparation of single cell suspensions from the popliteal lymph nodes draining the injection site, and for the isolation of these cells in micro-droplets have been described previously (Nossal, 1958, 1959*b*). Several changes were made in the present experiments, and these are included in the following abbreviated description.

Both popliteal lymph nodes from one animal were pooled, teased with needles in a Petri dish containing Earle's saline buffered to pH 7·0 with tris (hydroxy-methyl) amino methane (Tris, Sigma and Co.), forced through a coarse sieve, and gently washed three times by centrifugation. A small aliquot of the first deposit of cells was used for the preparation of air-dried smears of lymph node cells. The final deposit of cells was suspended in sufficient Earle's saline supplemented with 30 per cent normal rat serum to make the concentration approximately 10^6 to 5×10^6 cells per ml. A cover-slip was then covered with medicinal paraffin oil B.P. (H. C. Sleigh & Co.) and a large depot drop of cells was placed on it. About 40 large droplets containing suspending medium only were also deposited. The cover-slip was then inverted over a de Fonbrune (1949) type oil-chamber. By micromanipulation at 100-fold magnification (dark-ground), about 40 pairs of droplets of pure suspending medium containing about 10^{-5} to 10^{-6} ml. of fluid were deposited on known spots on the cover-slip. After about 10 min., the majority of the cells in the depot drop settled to the bottom of the drop, and could be surveyed and compared for differences in size, regularity and brilliance. With some experience, it became possible to predict with fair accuracy the histological type of any given cell at 100-fold magnification (dark-ground). The desired cell could then be drawn into the micropipette, washed in a clean large droplet of suspending medium, and deposited into one of the small droplets of suspending medium, together with a minimal quantity of the final washing fluid. A similar quantity of the final washing fluid was instilled into the second droplet of each pair, and this droplet acted as a control. This procedure was repeated as often as desired.

The oil-chamber was then incubated at 37° for 4 hr., and replaced on the microscope stage. Five to eight motile bacteria were instilled into each small droplet by micromanipulation. The droplets were surveyed for motility of the bacteria at frequent intervals, and if total loss of motility of all the bacteria occurred within 30 min. at room temperature, this was termed " inhibition ".

Titration of antibody content of microdroplets.—Droplets containing single cells showing inhibition were inoculated with a further 5–8 bacteria, and observed at frequent intervals. If immobilization of the second lot of bacteria occurred in any droplet, a third lot was introduced, and so on until the antibody content had been exhausted. The antibody titre of each droplet was expressed as the number of lots of bacteria immobilized, and recorded as 1, 1½, 2, 2½, etc. For this purpose, the figure ½ meant partial immobilization of the final lot of bacteria added. The final lot of bacteria was considered to be partially immobilized if 1 or 2 bacteria remained motile.

Serum titrations.—Serial 2-fold dilutions of the sera from the rats studied were made in physiological saline containing 1 : 100 nutrient broth and 1 : 100 of a 3–4 hr. broth culture of *Salm. adelaide* of standard turbidity. The concentration of organisms in this final mixture was about 10^6 per ml. Samples of each serum dilution were transferred onto a glass slide, under a layer of paraffin oil, and after 30 min. at room temperature were surveyed for motility of the organisms. Ninety per cent immobilization was taken as the end point, and the reciprocal of the serum dilution giving 90 per cent immobilization was termed the titre of the serum.

Staining of smears.—A small aliquot of packed lymph node cells was suspended in about 1–3 times its own volume of physiological saline supplemented with 10 per cent horse serum. A thin smear of this suspension was prepared on a cover-slip, dried by a rapid draught of air, and stained with Leishman's stain. Differential cell counts were made at 900-fold magnification. About 500–1000 cells from each animal were counted.

Staining of single cells.—Satisfactory staining of the single cells in their microdroplets proved difficult. Many conventional fixatives and stains were tried and found ineffective in this situation. The most satisfactory results were obtained when the cells were fixed and stained in the one manoeuvre by the introduction into the microdroplets of a small quantity of a stain of the following composition : Glacial acetic acid 55 ml., water 45 ml., Orcein 0·6 g., light green 0·2 g.

Solutions of orcein and light green in acetic acid were stored separately and mixed immediately before use to give the correct final concentration. These 2 dyes were chosen because

they act well at an acid pH. Best results were obtained when a small quantity of the dye was instilled into each microdroplet at some distance from the cell, and allowed to diffuse throughout the droplet. The cells were examined after 10–30 min. at room temperature, at 420-fold or 900-fold magnification.

RESULTS

Cell types seen in lymph nodes from immunized animals

In lymph nodes removed from animals 1–12 days after primary or secondary immunization, 5 classes of cells could be distinguished, namely lymphocytes, macrophages, blast cells, polymorphs and the cells of the plasma cell series. Further, numerous amorphous pink smudges representing squashed cells were seen. These were disregarded in the differential counts. The histological characteristics of these cells as observed in Leishman-stained smears were as follows :

Lymphocyte.—The smallest cell ; dark purplish-blue nucleus and large round masses of chromatin ; cytoplasm scanty, pale blue.

Macrophage.—Largest cell ; irregular outline ; cytoplasm packed with large round blue granules. Nucleus when visible is small, and placed anywhere in the cell.

Blast cell.—Large cell with large oval light reddish-purple nucleus and fine fibrillary chromatin pattern ; cytoplasm scanty, pale blue.

Polymorph.—Only rarely seen. Nearly all the polymorphs seen were eosinophils, with orange cytoplasmic granules and a U-shaped or annular nucleus with poorly defined lobulation.

Plasma cell series.—These cells are all larger than lymphocytes, and have an eccentric nucleus, a perinuclear clear zone, and dark blue cytoplasm. For convenience, we have divided them into three classes, but the division is arbitrary and the classes grade into each other.

Plasmablast.—Large cell, about the same size as blast. Similar to blast in many respects, but has a slightly denser nuclear chromatin pattern and more abundant darker cytoplasm ; slight nuclear eccentricity and perinuclear clear zone are apparent.

Immature plasma cell.—Somewhat smaller than plasmablast. Nucleus denser, smaller. Cytoplasm very abundant, dark blue and often foamy.

Mature plasma cell.—Somewhat smaller than immature plasma cell, but larger than lymphocyte. Nuclear chromatin very dense and clumped. Nucleus the size of a lymphocyte nucleus or smaller. Cytoplasm dark blue and less abundant than in immature plasma cell.

When the lymph node cells were stained in microdroplets by the orcein-light green stain, the chief features on which cell classification was based were retained. The nuclei stained various shades of red, and good nuclear detail was obtained. The fixation did tend to cause a little nuclear shrinking. The cytoplasm of blast cells and lymphocytes was stained a very light green, and the cytoplasm of plasmablasts and plasma cells a deeper green. The granules of the macrophages were also deep green, and those of the polymorphs light green. As optical conditions in the microdrops were far from ideal, it was considered unwise to attempt to grade plasma cells into mature and immature categories. The perinuclear clear zone so characteristic of plasma cells stained with Leishman's stain was

generally not seen. Single cells stained by orcein and light green are shown in Fig. 5–10.

Recognition of unstained cells

The histological characteristics of unstained lymph node cells could readily be appreciated using phase contrast microscopy at ×900 magnification. Unfortunately, phase optics were not available for the majority of these experiments. However, provided many lymph node cells were simultaneously surveyed at ×100 magnification (dark-ground), minor differences in the size, brilliance and regularity of the cells could be appreciated. After some practice, it soon became possible to predict the histology of unstained cells with about 80–90 per cent accuracy. The most important criterion was the size of the cells. Lymphocytes were much the smallest cells, plasma cells were somewhat larger and plasmablasts and blasts larger still. Macrophages were the largest of all the cells, and had as well a characteristic " frilly " outline.

Lymph node cells are notoriously difficult to maintain *in vitro* for any length of time. Even after four hours in our microdrops, some of the cells showed evidence of degeneration, such as cytoplasmic vacuolation and fragmentation, and nuclear pyknosis. This effect increased after the instillation into the droplets of bacteria. Accordingly some of the cells could not be accurately classified even after staining, and are listed as " unidentified ".

The results shown in Table I exemplify the degree of accuracy that can be expected when the cell type is predicted at 100-fold magnification. The table shows the correctness or otherwise of predictions made on cells picked out from a pool of lymph node cells derived from hyperimmunized animals.

TABLE I

Predicted histology of unstained cell		Observed histology of cell stained with orcein and light green	
Lymphocyte	35	Lymphocyte	32
		Plasma cell	2
		Unidentified	1
Plasma cell	34	Plasma cell	26
		Blast	3
		Lymphocyte	4
		Unidentified	1
Macrophage	4	Macrophage	4
Total	73	Correct	62
		Incorrect	9
		Unidentified	2

Histological changes during antibody production

Differential cell counts have been performed on Leishman-stained smears derived from the popliteal lymph nodes of 19 immunized and 2 normal animals (Table II, Fig. 1 and 2).

Primary response.—After primary immunization, there was an increased proportion of cells of the plasma cell series. Plasmablasts were first seen on the 5th day after immunization, when they reached their peak concentration of

16 per thousand, and were still present in increased numbers up to the 7th day. Immature plasma cells, first seen on the fifth day, reached their peak concentration

TABLE II.—*Proportions of Cell Types Present in Immunized Lymph Nodes (Cells per thousand)*

	Days*	Cell type: Lympho-cyte	Blast	Plasma-blast	Immature plasma cell	Mature plasma cell	Macro-phage	Eosino-phil
Primary antigenic stimulation	0†	993	2	0	0	0	5	0
	0†	979	16	2	1	1	1	0
	3	980	13	0	0	0	7	0
	5	967	6	16	3	0	7	1
	6	970	7	7	10	1	4	0
	7	938	14	9	18	15	5	0
	7	923	7	7	17	39	4	2
	9	955	17	0	8	12	4	4
	10	969	15	2	0	2	13	0
	12	984	2	0	2	3	6	2
Secondary antigenic stimulation	1	886	91	0	0	16	7	0
	2	911	46	34	4	0	5	0
	3	846	101	35	6	2	9	0
	4	788	49	54	94	11	5	0
	5	854	61	33	38	7	8	0
	6	932	7	0	13	37	9	0
	7	957	8	2	5	21	6	0
	8	979	11	0	1	6	1	0
	9	943	16	0	4	22	8	4
	12	988	3	0	0	6	3	0

* Days after injection of antigen.
† Unimmunized animals.

on the 7th day, and were still readily detectable on the 9th day. Mature plasma cells were first seen on the 6th day, reached their peak concentration of 27 per thousand on the 7th day, and a few were still detectable after 12 days. The proportion of blasts was too variable in the two normal pairs of nodes examined to allow of any conclusion to be drawn regarding their possible increase.

Secondary response.—The cellular response after secondary immunization is greater, and occurs earlier than after primary immunization. Also, blast cells increase significantly in the first 5 days after secondary immunization. On the first day after immunization, 91 blasts per thousand were seen and at 3 days 101 per thousand were present. The number of blasts present was still raised at 5 days. Plasmablasts were first seen 2 days after immunization, and reached their peak of 54 per thousand at 4 days. Very few were seen after 5 days. Immature plasma cells were also seen in small numbers at 2 days, and reached their peak of 94 per thousand at 4 days. They were rarely seen after 6 days. Mature plasma cells were seen in small numbers at 1 to 5 days, and reached their peak concentration of 37 per thousand at 6 days. Small numbers were still seen after 12 days.

It is emphasized that these figures represent proportions of cells present, and not total numbers. In fact there is a moderate enlargement of the nodes after primary immunization, and after secondary immunization a greater enlargement, which is maximal at 1 day and diminishes after the 4th or 5th day.

In all the thousands of cells surveyed, only 1 mitotic figure was seen, and this in a blast cell 4 days after secondary stimulation.

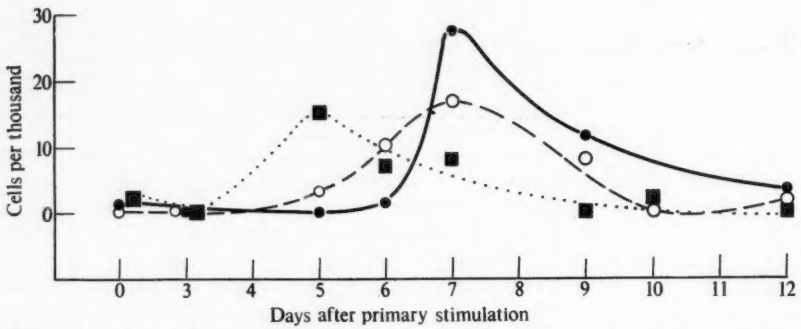


FIG. 1.—Proportions of various lymph node cell types after primary antigenic stimulation.
■ . . . . ■ — Plasmablasts. ○ — — — ○ — Immature plasma cells.
●——● — Mature plasma cells.

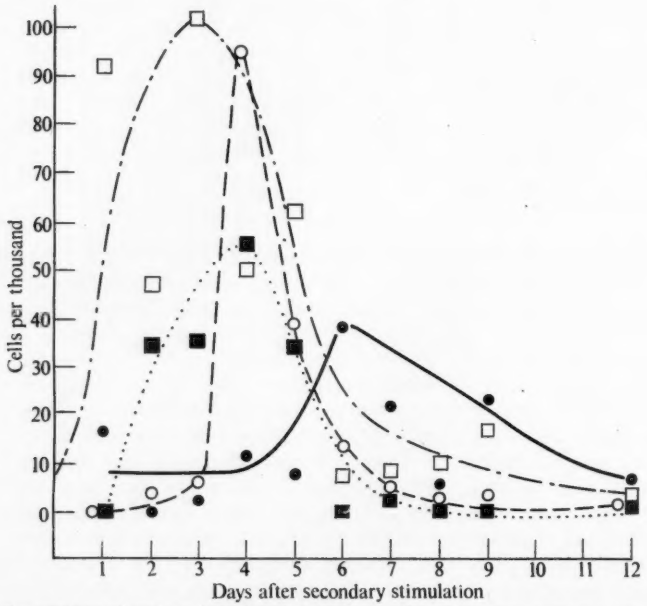


FIG. 2.—Proportions of various lymph node cell types after secondary antigenic stimulation.
□—·—□ — Blasts. ■ . . . . ■ — Plasmablasts. ○— — —○— Immature plasma cells.
●——● — Mature plasma cells.

Antibody production by various cell types

Antibody production by single cells of each of the above types has been determined in microdroplets (Tables III and IV). In the majority of the droplets,

only 5 bacteria were introduced to test for antibody production. In some of the control droplets up to 3 of these 5 bacteria were immobilized, presumably by small amounts of natural antibody in the normal rat serum of the medium, or by minute amounts of antibody left in the final washing fluid of the cells. Thus with only 5 bacteria as the test antigenic dose, one does run a risk of false positive results occurring if, for example, cells are inadequately washed.

TABLE III.—*Histology of Antibody-producing Cells in the Primary Response*

			Cell type						
Expt.	Days*	Serum titre	Plasma cells	Plasma-blasts	Blasts	Lympho-cytes	Macro-phages	Unidenti-fied	Totals
1	1	<10	0/7†	0/0	0/0	0/15	0/5	0/0	0/27
2	3	<10	0/1	0/0	0/1	0/17	0/0	0/2	0/21
3	5	120	1/5	0/5	0/0	0/14	0/0	0/1	1/25
4	6	160	0/9	0/5	0/2	0/15	0/4	0/0	0/35
5	7	2,400	8/21	1/2	0/1	0/11	0/1	0/0	9/36
6	7	2,560	9/15	1/2	0/1	0/9	0/1	0/3	10/31
7	8	9,600	5/14	1/3	0/0	0/12	0/0	0/3	6/32
8	9	1,200	4/8	0/0	0/1	0/20	0/1	1/4	5/34
9	10	4,800	0/4	0/1	0/3	0/18	0/2	0/0	0/28
10	12	24,000	1/6	0/0	0/1	0/15	0/4	0/0	1/26
	Total		28/90	3/18	0/10	0/146	0/18	1/13	32/295

* Days after primary antigenic stimulation.
† Numerator indicates number of active cells. Denominator indicates number of cells tested.
In Exp. 1 the animal received several injections of sheep red cells.

TABLE IV.—*Histology of Antibody-producing Cells in the Secondary Response*

			Cell type							
Exp.	Days*	Serum titre	Plasma cells	Plasma-blasts	Blasts	Lympho-cytes	Macro-phages	Poly-morphs	Uniden-tified	Totals
11	1	25,000	1/3†	0/0	0/15	0/15	0/1	0/0	0/1	1/35
12	2	12,800	0/1	1/6	0/7	0/21	0/0	0/0	0/3	1/38
13	3	4,800	1/4	2/7	0/13	0/15	0/0	0/0	0/4	3/43
14	4	12,800	12/18	2/8	0/1	1/18	0/1	0/0	1/5	16/51
15	5	100,000	13/18	0/3	0/4	0/13	0/0	0/0	0/6	13/44
16	6	500,000	8/11	0/1	0/2	0/19	0/0	0/0	1/5	9/38
17	7	160,000	4/7	0/0	0/3	0/11	0/0	0/0	1/2	5/23
18	8	64,000	10/14	0/0	0/4	0/14	0/0	0/1	2/6	12/39
19	9	640,000	5/12	0/0	0/5	0/15	0/1	0/1	0/6	5/40
20	12	30,000	1/9	0/0	1/2	0/19	0/2	0/0	0/2	2/34
	Total		55/97	5/25	1/56	1/160	0/5	0/2	5/39	67/385

* Days after secondary antigenic stimulation.
† Numerator indicated number of active cells. Denominator indicates number of cells tested.

In Exp. 1, lymph node cells derived from an animal immunized with several injections of sheep red cells were examined, in order to ensure that plasma cells did not liberate a substance capable of non-specifically immobilizing the test bacteria. In Exps. 2–10, 268 single cells derived from primarily stimulated animals were examined. Thirty-two of these produced demonstrable antibody; of these 28 were plasma cells, 3 were plasmablasts, and one was unidentified.

At the height of the primary response (7–9 days), 26 out of 58 (45 per cent) plasma cells were producing demonstrable antibody. In Exps. 11–20, 385 cells derived from secondarily stimulated animals were examined. Sixty-seven of these produced demonstrable antibody ; of these 55 were plasma cells, 5 were plasmablasts, 5 were unidentified, 1 was a blast and 1 was a lymphocyte. The titres of the blast cell and the lymphocyte were respectively $1\frac{1}{2}$ and 1. During the most active phase of the secondary response (4–8 days) 47 out of 68 (69 per cent) plasma cells were producing demonstrable antibody.

In certain droplets, some bacteria adhered to the surface of the cell, and on rare occasions when only partly immobilized actually dragged the cell around the microdroplet. This phenomenon of adherence was systematically studied in Exps. 4–20. It was recorded as positive 76 times ; 46 times the bacteria adhered to active cells, of which 42 were plasma cells and 4 were unidentified, and 30 times they adhered to cells which had not produced sufficient antibody to immobilize completely the 5 bacteria instilled. Of these, 24 were plasma cells, 2 were plasmablasts, 1 was a lymphocyte and 2 were unidentified.

Amount of antibody produced by single plasma cells and plasmablasts

The titre of antibody in the droplets is shown in Fig. 3 and 4.

In each response, a mean has been calculated of all titres of 1 or greater produced by plasma cells. In the primary response, this was 2·2 and in the secondary response 6·3, which is a barely significant difference as analyzed by "Student's" t test ($0{\cdot}05 > p > 0{\cdot}02$). In the primary response, the mean of the positive titres produced by single plasma cells was not significantly different between 7, 8 and 9 days. In the secondary response the titres were not significantly different between any of the days 4, 5, 6, 7, 8 and 9.

DISCUSSION

The essential new technique which has been used in this work is the determination of the cell type of living unstained cells. This has been possible after considerable study of the correlation of unstained appearances with the appearance of the same cell stained with orcein and light green. The accuracy of prediction of cell type is about 90 per cent. Our results confirm the generally held view that the chief, if not the sole producers of antibody are the cells of the plasma cell series. Out of 93 antibody-producing cells of identifiable histology, 83 were plasma cells and 8 plasmablasts. Of the 43 plasmablasts examined, only 8 made antibody and of these 6 had titres of 2 or less. Plasmablasts were less active antibody-producers than plasma cells. The staining method employed for single cells did not enable us to distinguish confidently immature from mature plasma cells. As the mean titre of antibody produced by single cells early in the immune response, when immature plasma cells predominated, did not differ significantly from the mean titre late in the immune response when mature plasma cells predominated, there does not appear to be much difference in the average amount of antibody produced *in vitro* by individual immature and mature plasma cells. However, far more cells will have to be tested before this point can be regarded as proven.

The finding of 1 active lymphocyte out of 306 tested, and of 1 active blast out of 66 tested is difficult to explain. In view of the fact that we were working so

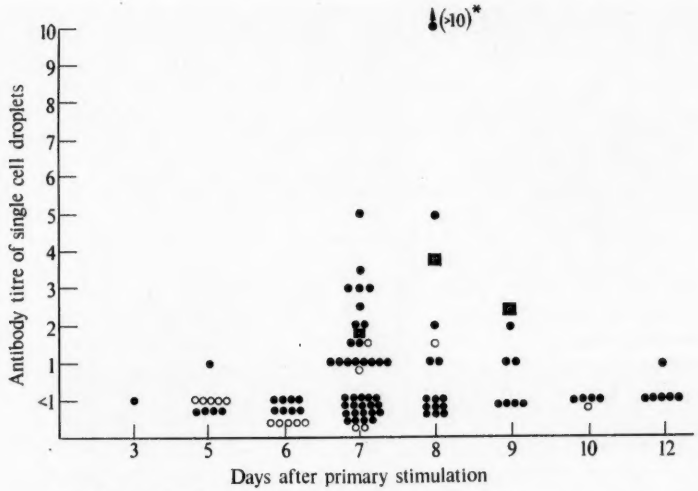


FIG. 3.—Antibody titres produced by single cells: Primary response.
○ — Titres of plasmablasts. ● — Titres of plasma cells. ■ — Mean titres of plasma cells.
* For statistical purposes this titre was taken as 10.

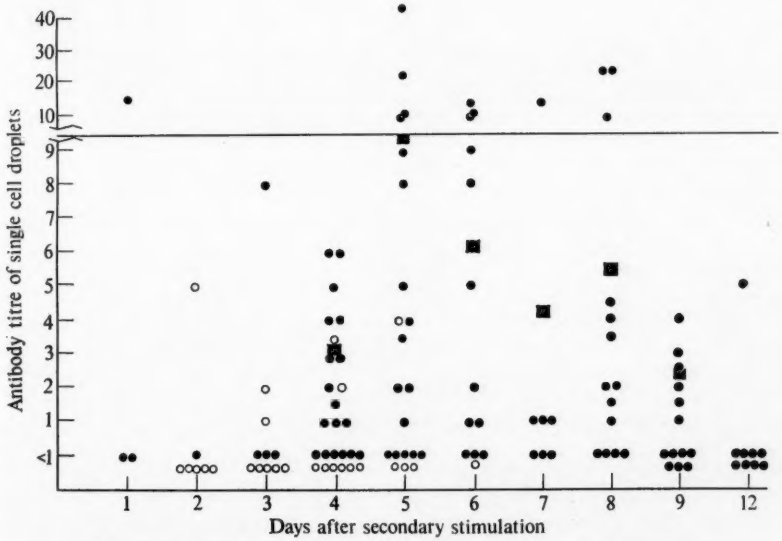


FIG. 4.—Antibody titres produced by single cells: Secondary response.
○ — Titres of plasmablasts. ● — Titres of plasma cells. ■ — Mean titres of active plasma cells.

close to the threshold of the test, it is tempting to assume that these results were due to some technical error such as inadequate washing of the cells. Other explanations are also possible. The active lymphocyte may have been a plasma cell which had lost most of its cytoplasm during centrifugation or incubation; the blast cell may in fact have been on the way to becoming a plasma cell.

None of the 23 macrophages or the 2 polymorphs tested produced demonstrable antibody.

The finding that bacteria adhered to the surface of some of the plasma cells is in agreement with the findings of Reiss, Mertens and Ehrich (1950) and of Moeschlin and Demiral (1952). The most likely explanation of this phenomenon seems to be that the plasma cells concerned have on their surface either bound antibody or at least configurations complementary in pattern to bacterial antigenic determinants. It is possible that those plasma cells exhibiting adherence but not forming detectable antiflagellar antibody, were in fact producing antibody to traces of contaminating somatic material in our antigen.

Both the results of the single cell studies and the differential counts of Leishman-stained lymph node cell smears are in agreement with the findings of Leduc, Coons and Connolly (1955) who studied the histology of antibody production by the fluorescent technique. However, these authors did not comment on the presence of very large numbers of primitive blast cells as early as 1 day after secondary immunization. Moreover, they reported frequent mitotic figures 2–3 days after secondary immunization, while we saw no mitotic figures among over 1000 cells examined at this period. The one mitotic figure we did see occurred 4 days after secondary immunization.

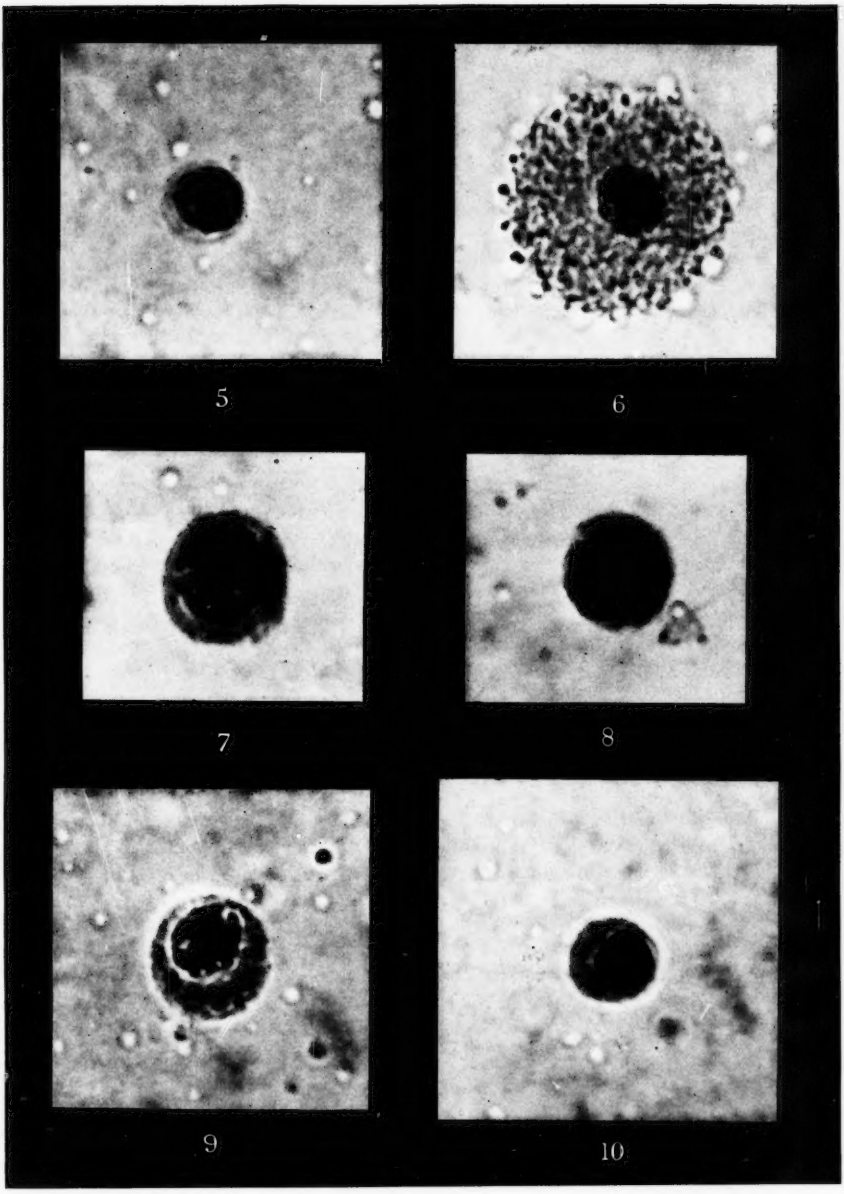
We consider the marked increase in blast cells occurring very soon after secondary immunization to be of great interest. Insufficient attention has been drawn in the literature to the fact that the earliest histological response to secondary immunization is the appearance of large numbers of these cells, which are histologically indistinguishable from the primitive cells present in every normal lymph node. A similar but smaller increase in blast cells may occur after primary immunization; we have not examined sufficient normal and primarily immunized nodes to establish this point. The enlargement of the popliteal lymph nodes 1 day after secondary stimulation is so marked that it is probably not solely due to division of the cells present prior to stimulation. Probably some of the increase is due to the attraction of cells to the lymph node from the "mesenchymal reserve". Our results seem consistent with the view that mature plasma cells are derived from lymphocytes via the stages blast, plasmablast and immature plasma cell.

In view of the wide scatter of antibody titres obtained in our single cell studies, it is difficult to be certain how far the amount of antibody formed by a plasma cell *in vitro* corresponds to the actual rate of synthesis *in vivo*. It is possible that the variation in titre mirrors mainly the differences in the degree of damage suffered by plasma cells during centrifugation, manipulation and incubation in

EXPLANATION OF PLATE.

FIGS. 5–10.—Single lymph node cells from immunized animals stained with Orcein and Light Green photographed at × 700 magnification and enlarged approximately another four-fold.

Fig. 5: Lymphocyte. Fig. 6: Macrophage. Fig. 7: Blast cell. Fig. 8: Plasmablast. Fig 9: Plasma cell, probably immature. Fig. 10: Plasma cell, probably mature.



Nossal.

such abnormal surroundings. It is also possible that the low titres (1–2) represent passive release of stored antibody, and the higher titres various degrees of actual synthesis of antibody *in vitro*. However, it does appear that under the conditions of our experiments, plasma cells removed during the secondary response make more antibody than plasma cells removed during the primary response. These findings confirm and extend the results presented in the previous paper of this series (Nossal, 1959*b*).

The ability to recognize the cell type of living cells has enabled this work to be done more rapidly than would have been possible by earlier techniques. With this technique, it will be possible in future to concentrate attention entirely on the study of plasma cells, with the result that more antibody-producing cells will be found in any one experiment than previously. This should prove particularly useful in a more detailed analysis of the phenotypes of antibody-producing cells following immunization with two or more antigens.

SUMMARY

The techniques for the detection of antibody production by single cells previously described have been applied to the problem of the histological characteristics of antibody-producing cells. Antibody-production *in vitro* has been studied in 653 single cells from primarily or secondarily stimulated animals. All cells were subsequently stained with orcein and light green. Out of 601 cells that could be classified histologically, 93 formed detectable amounts of antibody, and of these 91 were plasmablasts or plasma cells. This led to the conclusion that cells of the plasma cell series were the main, if not the sole producers of antibody.

The amount of antibody produced by individual plasma cells varied within wide limits, but was higher on the average in the secondary response.

Examination of Leishman-stained smears of lymph node cells showed that after secondary immunization, the cellular response was greater and occurred earlier than after primary immunization.

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THE LIPOLYTIC ACTIVITY OF HEART AND AORTA IN EXPERIMENTAL ATHEROSCLEROSIS IN RABBITS

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IT has been previously shown (Zemplényi and Grafnetter 1958; Zemplényi, Lojda and Grafnetter 1958) that the lipolytic activity of the aorta, estimated in terms of liberated fatty acids, of species susceptible to experimental atherosclerosis is low in comparison with that of rat aorta. The latter animal is known to be relatively resistant to the ordinary techniques of producing experimental atherosclerosis. The possibility of rapid lipid deposition in tissues with low lipolytic activity has also been previously suggested.

The present communication deals with the question of whether, and to what extent, there are functional adaptations in the lipolytic activity of rabbit aorta in chronic cholesterol feeding of experimental animals. The lipolytic activity of the myocardium was investigated at the same time since it is known to be high (Zemplényi and Grafnetter 1958).

MATERIALS AND METHODS

Experimental atherosclerosis was produced in 18 male rabbits aged about 6 months, by feeding 1 g. of cholesterol dissolved in 10 g. margarine or soy oil daily for 6 months. Nine male animals of the same age, kept on a standard diet, were used as controls.

The animals were killed by air embolism and the extent of atherosclerotic changes in the aorta was assessed by a method previously described (Fodor, Zemplényi, Lojda and Felt 1958), employing a five degree scale for expressing the severity of tissue damage, as follows:

1. Changes can be established solely by microscopy (slight thickening of intima with mucoid infiltration, slight intracellular and extracellular deposition of lipids. "Faserzellen"–Benninghoff,—sparsely dispersed foam cells).

2. Alterations in the ascending aorta and aortic arch in the form of small round or oval yellowish grey foci (1–2 mm. in diameter) at the intimal surface. Microscopically there is a clear accumulation of foam cells.

3. In the ascending aorta and aortic arch small plaques mainly in the parts adjacent to the origin of the great vessels.

4. Plaques form in the descending aorta (mainly on the dorsal side and near the intercostal arteries). Confluent plaques of the ascending aorta are visible.

5. Large confluent plaques also seen in the descending aorta with foci of foam cells located in the plaques, chiefly in the deeper parts of the intima.

Lipolytic activity of tissues was estimated by a method previously described (Zemplényi and Grafnetter 1958), consisting of incubation of lipaemic human serum, diluted 1 : 1 with Sörensen phosphate buffer at pH 7·35, with a weighed amount of myocardial *brei* or finely minced aorta that had been carefully freed from periarterial tissue and adventitia. The extent of lipolysis was expressed by the amount of liberated fatty acids in μM per g. of either fresh, dry, or fat free dry, tissue.

RESULTS

Fig. 1 shows the amount of fatty acids (per g., of fresh tissue) liberated by aortae of normal and atherosclerotic rabbits irrespective of the extent of atherosclerotic changes. The lipolytic activity of atheromatous rabbit aorta is significantly higher than that of normal animals within 95 per cent confidence limits.

Fig. 2 demonstrates the relation between the degree of atherosclerosis and the lipolytic activity of aortae on a wet weight basis. It can be seen that there is a

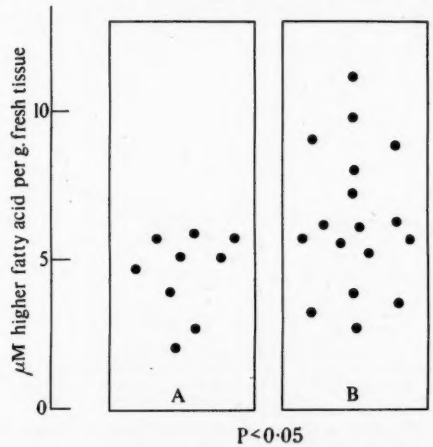

FIG. 1.—Comparison of lipolytic activity of 17 atheromatous rabbit aortae (irrespective of degree of atherosclerotic alterations) with lipolytic activity of the aortae of control animals. Aortic tissue incubated with continuous shaking with 2 ml. of substrate at 37° for 150 min. (Substrate: human male lipaemic serum diluted with an equal part of Sörensen phosphate buffer, pH 7,38). A: control animals, B: experimental animals.

linear relation (correlation coefficient r = 0·8087) at a probable error of less than 0·001. There is a still closer approach to linearity (r = 0·8469) if slighter degrees of atherosclerotic alterations (below 2·5, according to our scale) are excluded.

Figs. 3 and 4 show that basically the same results are obtained by relating lipolytic activity to unit weight of dry or fat free dry tissue.

Further analysis of results, as borne out by Fig. 5, shows the lipolytic activity of aortae (wet weight basis) of normal animals to differ very significantly from that of animals with atherosclerosis (degree 3–5), at $P < 0{\cdot}001$. The lipolytic activity of the aortae of normal rabbits and atherosclerotic animals with vascular changes below degree 3 do not differ. The same results are obtained when basing calculations on dry or fat free dry tissue.

Fig. 6 shows that the amount of fatty acids liberated from lipaemic serum by myocardial tissue is higher in atherosclerotic rabbits than in the animals of the control group.

DISCUSSION

It is beyond doubt that the atherosclerotic vessel contains more lipid substance than the normal vessel, irrespective of the pathogenetic mechanism of ather-

sclerosis. However, the problem of the more detailed mechanism of lipid deposition in the vascular wall has attracted only slight attention in the past. Practically no attention has been paid to the aorta as an organ capable of synthesizing, accumulating and removing vitally important substances including lipids (Holman, McGill, Strong and Geer, 1957).

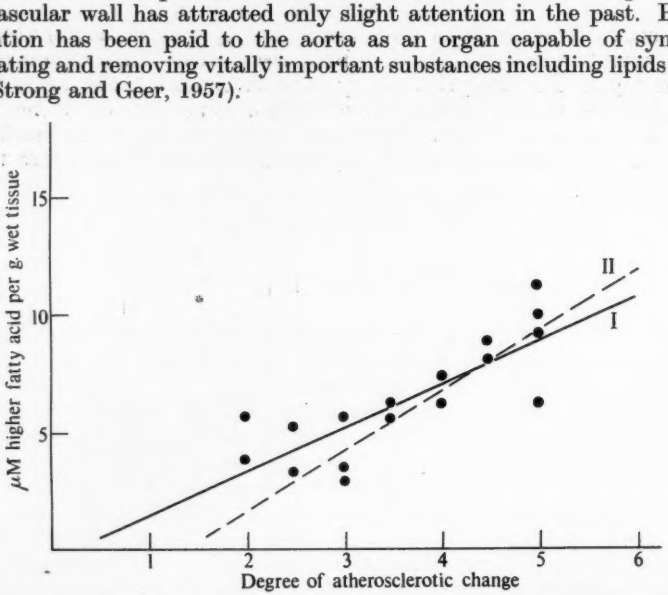

FIG. 2.—Correlation of lipolytic activity of aorta (wet weight basis) with degree of atherosclerotic alterations.
Regression line I (for degrees of atherosclerotic alteration 0–5): $y_i = -0{\cdot}31 + 1{\cdot}81\,x_i$, $r = 0.8087$, $P < 0{\cdot}001$. Regression line II (for degrees of atherosclerotic alterations 3–5): $y_i = -3{\cdot}41 + 2{\cdot}52\,x_i$, $r = 0{\cdot}8469$, $P < 0{\cdot}001$.

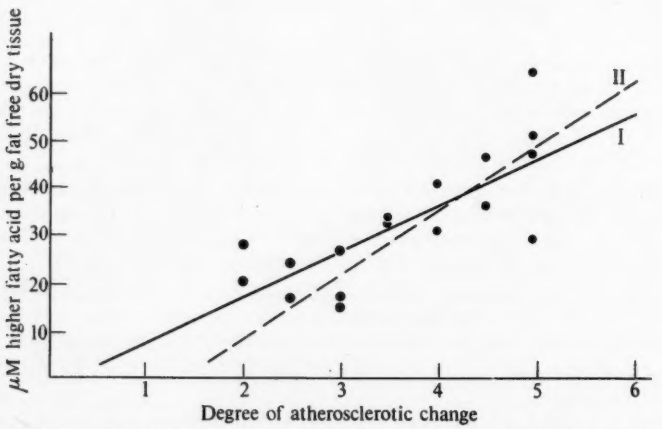

FIG. 3.—Correlation of lipolytic activity of aorta (fat free dry weight basis) with degree of atherosclerotic alterations.
Symbols as in Fig. 2. Regression line I: $y_i = -3{\cdot}48 + 9{\cdot}93\,x_i$, $r = 0{\cdot}7842$, $P < 0{\cdot}001$. Regression line II: $y_i = -18{\cdot}7 + 13{\cdot}42\,x_i$, $r = 0{\cdot}7902$, $P < 0{\cdot}01$.

The development in rabbits of functional adaptation of the lipolytic enzyme system due to chronic feeding of cholesterol and fat is one of the main results of the experiments presented here. The myocardium is an organ with the highest lipolytic activity which was found to be considerably increased in our experiments.

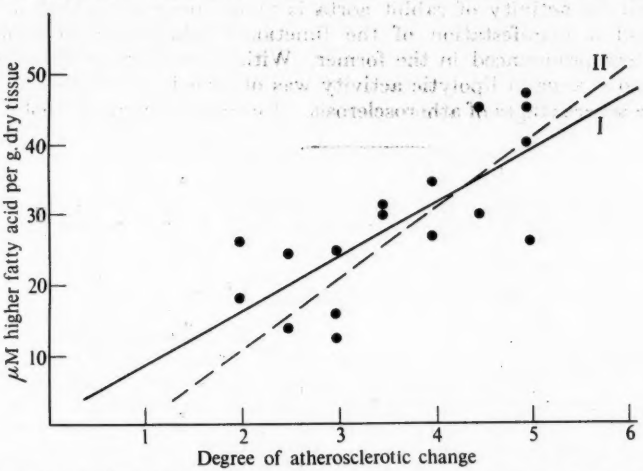

FIG. 4.—Correlation of lipolytic activity of aorta (dry weight basis) with degree of atherosclerotic alterations.
Symbols as in Fig. 2. Regression line I: $y_i = 1{\cdot}88 + 7{\cdot}48\,x_i$, $r = 0{\cdot}7471$, $P < 0{\cdot}001$.
Regression line II: $y_i = -9{\cdot}46 + 9{\cdot}97\,x_i$, $r = 0{\cdot}7510$, $P < 0{\cdot}01$.

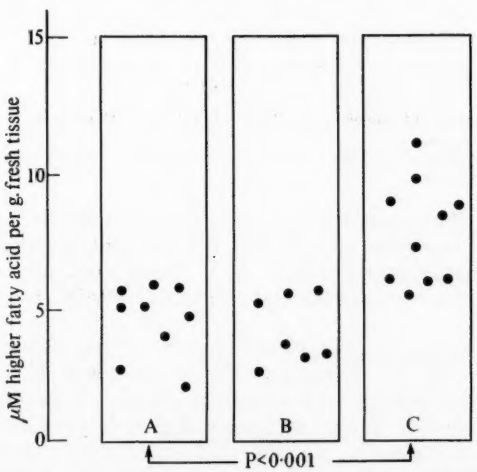

FIG. 5.—Lipolytic activity of aorta in individual groups of severity of atherosclerotic alterations.
A: control animals, B: animals with degree of atherosclerotic alterations 0–2·5, C: animals with degree of atherosclerotic alterations 3–5. Experimental conditions as in Fig. 1

This is evidently of significance for the utilisation of excess fat fed to these animals and for myocardial metabolism which, according to Bing (1955), Gordon and Cherkes (1956) and others, is capable of covering its energetic requirements to a large extent with the higher fatty acids.

The lipolytic activity of rabbit aorta is much lower than that of the myocardium and a manifestation of the functional adaptation to cholesterol-fat feeding is less pronounced in the former. With lesser degrees of atherosclerotic alteration no change in lipolytic activity was observed; the latter appears only in the more severe stages of atherosclerosis. However, this raised lipolytic activity

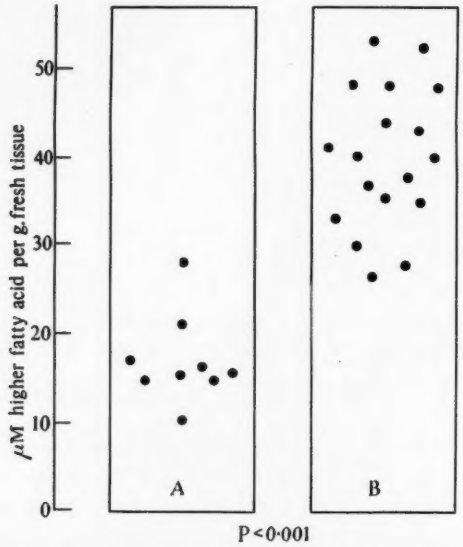

FIG. 6.—Lipolytic activity of myocardium of 18 male rabbits with experimental atherosclerosis, and of 9 control animals.
Experimental conditions as in Fig. 1. A = control animals. B = experimental animals.

is far below the lipolytic activity of normal rat aorta which, according to simultaneous comparative experiments, is still 100 per cent in excess of the maximal activity of the atherosclerotic rabbit aorta. Functional adaptation of lipolysis in rabbit aorta due to increased fat and cholesterol intake is evidently of a rather low order.

The absence of changes of lipolytic activity in less severe stages of atherosclerosis cannot be directly accounted for on the basis of the present results. Our histochemical investigations of esterolysis (Lojda and Zemplényi, 1958) reveal the intima to be the site of enzymatic activity in the vessels of atherosclerotic rabbits. From this it can be inferred that enhanced lipolytic activity in the advanced stages of atherosclerosis is most probably linked with the pronounced intimal thickening of such vessels.

As already stated above, it is felt that, in tissues with low lipolytic activity, conditions exist for the deposition of triglycerides and cholesterol, the latter being

at least partly maintained in solution by triglycerides. The present results are in accord with such an idea.

SUMMARY

Chronic cholesterol feeding of rabbits leads to functional adaptation of the lipolytic enzyme system. The lipolytic activity of myocardial tissue is highly increased. Manifestation of functional adaptation of aortic tissue is less pronounced, becoming more significant only in the more severe stages of atherosclerotic change. The lipolytic activity of normal rat aorta is still 100 per cent in excess of the maximal activity of atherosclerotic rabbit aorta.

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IN VIVO GLYCOGENESIS IN X-IRRADIATED RATS

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THE observation of North and Nims (1949) on the increase in the liver glycogen levels in male rats following X-irradiation has led several workers (Ord and Stocken, 1953; DuBois and Petersen, 1954; Hevesy and Forssberg, 1955; Holmes, 1957), to study the mechanism of action underlying this phenomenon. Ross and Ely (1951) have shown that shielding of the liver or the pituitary did not have any influence on the deposition of glycogen. Denson, Gray, Gray, Herbert, Tew and Jensen, (1953) ascribed the glycogen accumulation following X-irradiation to retarded glycogenolysis, while the studies of McKee (1952) and Morehouse and Searcy (1955) indicated that the enhanced glyconeogenesis is the cause of an increase in glycogen in X-irradiated rats. Since it is known that cortisone increases the glycogen accumulation in liver, several investigators (Ross and Ely, 1951; Nims and Sutton, 1954; Bacq and Alexander, 1955; McKee and Brin, 1956; Cronkite and Bond, 1956) have considered the possibility that an increased level of liver glycogen in irradiated animals might be attributable to the involvement of adrenal glands. In view of these varied observations, carefully controlled experiments were necessary to determine whether the immediate glycogen accumulation observed in X-irradiated rats is due to a block in the utilization of glycogen or whether it is due to the stimulation of the synthetic processes leading to the formation of glycogen. This paper deals with these studies and in addition reports data on the influence of total body X-irradiation on levels of " free " and " bound " fractions of glycogen and on the *in vivo* rate of glycogenesis in liver and muscle of young rats. The latter has been studied by the method of Longley, Shirkey, Alvig and Roe, (1955) in which the amount of glycogen formed, following an intraperitoneal injection of a test dose of glucose is taken as a measure of the rate of glycogenesis.

MATERIALS AND METHODS

The animals used were male Wistar albino inbred rats, 2–2½ months old and weighing 150–200 g. They were maintained on a standard laboratory diet and water *ad libitum*. The rats were given a LD_{50} dose of 600r of total body X-irradiation (Smith, Patt, Tyree and Straube, 1950; Buchanan, Darby, Bridgforth, Hudson and Efner; 1953). A " G.E. Maximar" deep therapy unit, operated at 220 kV. and 10 m. amp. with 0·5 mm. Cu and 1·0 mm. Al filters was used. Irradiation was carried out on rats kept at a distance of about 35 cm. from the target of the X-ray tube and the dorsal and ventral sides were given 300 r each at a rate of 50 r per min. in air as determined by Victoreen dosimeter. Non-irradiated control animals were submitted to sham irradiation procedures for the same period of time.

The rats were divided into several groups of 6 animals each and were used in the following 3 experiments. In the first experiment the levels of total glycogen were determined in the liver and muscle of control and irradiated animals. Non-irradiated control animals were

killed after 0, 24 and 48 hr. of fasting. Irradiated animals were killed 24 hr. after irradiation and were fasted for 24 hr. before and 24 hr. after irradiation. In the second experiment free and bound fractions of glycogen were determined in the liver and muscle of the fed and the 48 hr. fasted controls and in the fasted irradiated animals. The third experiment was designed to study the *in vivo* rate of glycogenesis after intraperitoneal administration of glucose. The 48 hr. fasted controls and irradiated animals were given intraperitoneal injections of a 10 per cent solution of glucose in water at a level of 2 g. glucose per kg. body weight. Glucose was injected 3 hr. before death because this duration showed maximum glycogen synthesis. Another set of control and irradiated animals received normal saline in place of glucose and was used as the corresponding control for glucose injected animals. The average glycogen value of saline injected animals was subtracted from the average glycogen value of glucose injected animals and the difference was taken as an index of the rate of glycogenesis.

Rats in all the experiments were killed by decapitation and liver and skeletal muscle weighing about 1 g. were quickly removed, blotted free of the adhering blood and immediately chilled in ice-salt mixture. For the determination of the total glycogen, the chilled tissues were weighed and homogenized with an all glass homogenizer (Potter and Elvehjem, 1936) in cold 80 per cent methanol. The homogenates were centrifuged at 3000 r.p.m. in cold for 15 min. The supernatants containing free glucose and glucose phosphates were discarded and the residues were washed twice with 80 per cent methanol to remove the remaining traces of glucose. Glycogen was extracted from the residues by heating with 10 ml. of 5 per cent trichloroacetic acid (TCA) solution containing 0·1 per cent silver sulphate, at 100° for 15 min. (Kemp and Kits Van Heijningen, 1954). The tubes were then centrifuged at 3000 r.p.m. and 2 ml. samples of the clear supernatant were heated in wide test tubes at 100° with 6 ml. of 96 per cent sulphuric acid (Merck's) for 6½ min. (Mendel, Kemp and Myers, 1954). Hot sulphuric acid hydrolyses glycogen to glucose, which in subsequent stages of reaction develops a pink colour. The test tubes were then cooled under tap water and the intensity of the colour, which is stable for several hours at room temperature, was measured at 540 mμ in a Klett Summerson photoelectric colorimeter. The glycogen content in tissues was expressed in terms of glucose equivalents.

The separate determinations of free and bound forms of glycogen were carried out by differential extraction of the methanol washed residue with TCA reagent (Kemp and Kits Van Heijningen, 1954 ; Kits Van Heijningen and Kemp, 1955). For this purpose the residue made free of glucose by methanol washing was treated with 10 ml. of cold TCA reagent and centrifuged in cold at 3000 r.p.m. for 15 min. The clear supernatant contained all the free glycogen. The remaining residue was then washed once with TCA reagent to remove any traces of free glycogen and then heated for 15 min. at 100° with another 10 ml. portion of the TCA reagent and centrifuged. The supernatant from this operation contained the bound glycogen. Free and bound glycogen were then estimated by the procedure for total glycogen described above.

RESULTS AND DISCUSSION

The levels of total glycogen in the liver and muscle of control and irradiated animals are given in Table I. The data presented indicate that the total body X-irradiation in rats, which were fasted for 48 hr. (24 hr. before and 24 hr. after irradiation) resulted in a considerable increase in liver glycogen as compared to non-irradiated rats fasted for the same length of time. No such changes were noticed in the levels of muscle glycogen. This observation is in agreement with that of Kay and Entenman (1956) who observed that in fasting rats, whole body X-irradiation resulted in a sharp rise in liver glycogen, within 24 hr. It is interesting to note that the percentage of glycogen in the liver of fasted irradiated rats was much less than that found in normal fed animals. It is further evident from the table that when normal animals were fasted, the hepatic glycogen was considerably depleted within the first 24 hr. and was followed by a slight but significant increase during the next 24 hr. of fasting. The fact that rats were depleted of liver glycogen at the time of irradiation exposure and that it increased during the post-irradiation

period suggests that liver glycogen in X-irradiated animals was formed by augmentation of the synthetic processes and not by inhibition of the breakdown process. The data thus clearly indicated that the liver of X-irradiated animals showed an increased ability to synthesize and store glycogen.

TABLE I.—*Levels of Total Glycogen in Liver and Muscle of Control and X-irradiated Rats*

Group	Period of fasting (hr.)	Amount of glycogen as mg. glucose/100 g. wet tissue	
		Liver	Muscle
Controls			
Sham irradiated rats	0	4281·9±151·0*	468·5±17·4
	24	135·1±16·3	345·8±17·2
	48	332·8±33·6	198·9±26·0
X-irradiated rats	48†	1261·3±81·5	204·1±24·5

* Standard error of the mean.
† 24 hr. prior to and 24 hr. after irradiation.

Recent investigations (Bloom, Lewis, Schumpert and Shen, 1951 ; Van der Vies, 1954 ; Merrick and Meyer, 1954 ; Russell and Bloom, 1955 ; Gyermek and Fekete, 1955 ; Kits Van Heijningen and Kemp, 1955) indicated that tissue glycogen is built up of 2 physiologically different components, which make up a constant and reproducible proportion of the total glycogen. One of these is " free " and can be extracted with cold trichloroacetic acid. The other is " bound " and it is necessary to heat the suspension with trichloroacetic acid (Kemp and Kits Van Heijningen, 1954 ; Kits Van Heijningen and Kemp, 1955) or to destroy the tissue by alkaline hydrolysis (Bloom *et al.* 1951 ; Russell and Bloom, 1955) before the protein fixed glycogen can be brought into solution. Bloom and Knowlton (1953), Russell (1953), Merrick and Meyer (1954) and Russell and Bloom (1955) have described the " free " form of glycogen as labile, since alterations in the glycogen levels due to varying physiological conditions, are mainly associated with this fraction, in contrast to the " bound " glycogen which remains more or less constant. In view of this, it was of additional interest to determine the changes in these 2 fractions of glycogen following X-irradiation. The levels of total, " free " and " bound " glycogen in liver and muscle of control and irradiated animals are recorded in Table II. It is seen that the proportion of the free glycogen

TABLE II.—*Levels of " Free " and " Bound " Fractions of Glycogen in Liver and Muscle of Normal and X-irradiated Rats*

Group	Liver glycogen as mg. glucose/100 g. wet tissue			Ratio of free to total glycogen × 100	Muscle glycogen as mg. glucose/100 g. wet tissue			Ratio of free to total glycogen × 100
	Total	Free	Bound		Total	Free	Bound	
Control fed rats	4281·9	3535·0	946·9	82·6±2·45	468·5	297·5	171·0	61·4±2·65
Control, 48 hr. fasted rats	332·8	27·2	305·6	8·18±0·9	198·9	15·3	183·6	7·68±0·8
X-irradiated and fasted (24 hr. before and 24 hr. after irradiation)	1261·3	94·2	1167·1	7·50±1·1	204·1	17·2	186·9	8·44±0·9

differs in the 2 tissues, it is 82·6 per cent in liver and 61·4 per cent in muscle of normal fed animals. During fasting the free glycogen in both these tissues disappears much more rapidly than the bound form. Thus the free glycogen in liver decreases from 82·6 per cent of the total glycogen to 8·18 per cent after 48 hr. fasting, while in muscle it decreases from 61·4 per cent to 7·68 per cent. The bound glycogen, however, does not undergo such changes during fasting. This seems to be in agreement with the observations of Bloom *et al.* (1951), Merrick and Meyer (1954) and Russell and Bloom (1955). It is further seen that in X-irradiated rats, the rise in the liver glycogen levels is equally shared by the free and bound fractions, because their ratio in normal fasted rats remains the same even after irradiation exposure.

Tissue glycogen is known to be synthesized either from glucose (glycogenesis) or from non-carbohydrate precursors (glyconeogenesis) (Soskin and Levine, 1952 ; Stetten and Topper, 1955 ; Noggle, 1957). It, therefore, raises the question whether the increased hepatic glycogen found in X-irradiated rats results from increased glycogenesis or glyconeogenesis, or both. The fact that the pituitary-adrenal system is stimulated following total body X-irradiation (Nims and Sutton, 1954 ; Cronkite and Bond, 1956) and that it has an influence on glyconeogenesis (Long, 1942 ; White, 1950) has led McKee and Brin (1956) and Kay, Harris and Entenman, (1956) to conclude that enhanced glyconeogenesis of the carbon fragments resulting from the breakdown of tissue proteins, is an important source of hepatic glycogen in X-irradiated animals. This concept was further supported by the observations of increased transaminase activity in liver (Brin and McKee, 1956) and the increased incorporation of acetate-^{14}C into liver glycogen of X-irradiated rats (Morehouse and Searcy, 1955). However, little was known about the process of glycogenesis in X-irradiated rats. This aspect has been investigated here and the data on the *in vivo* glycogen synthesis from glucose in liver and muscle of normal and X-irradiated rats are presented in Table III. It will be apparent from this Table that when glucose is made available for glycogenesis, the glycogen synthesis takes place predominantly in the liver and not so much in the muscle. It is further seen that the glycogen synthesized by liver of X-irradiated rats, is about 61·8 per cent more than the control animals, suggesting thereby that the livers

TABLE III.—*Influence of X-irradiation on the* in vivo *Synthesis of Glycogen from Glucose (Glycogenesis) in Liver and Muscle of Fasted Rats**

	Amount of glycogen as mg. glucose/100 g. wt tissue			
	Liver		Muscle	
Group	Control rats	X-irradiated rats (24 hr.)	Control rats	X-irradiated rats (24 hr.)
Initial levels (saline injected rats)	308·2±24·0	1145·0±67·7	212·7±29·4	209·8±22·4
Levels after the administration of glucose† (glucose injected rats)	2033·7±158·0	3936·0±176·0	255·1±20·4	249·6±23·3
Net synthesis of glycogen in 3 hr.	1725·5	2791·4	42·40	39·80

* Control and irradiated animals were fasted for 48 hr. (In the irradiated group 24 hr. before and 24 hr. after irradiation.)

† Animals were injected intraperitoneally 2 g. glucose per kg. body weight 3 hr. prior to sacrifice.

of X-irradiated animals have a greater ability to synthesize glycogen from glucose. This observation is in accord with that of Baxi and Sahasrabudhe (1957) who demonstrated that in a glucose containing medium, the *in vitro* glycogen synthesis is greater with liver homogenates of X-irradiated than that of normal rats.

SUMMARY

Studies on the levels of total glycogen in fasted and fed normal animals and in fasted irradiated rats indicate that total body X-irradiation of 600 r increases the hepatic glycogen in fasted rats and that this accumulation of liver glycogen is due to increased synthetic activity rather than due to a block in the breakdown of glycogen.

Measurement of " free " and " bound " fractions of glycogen in liver and muscle of control and X-irradiated rats indicate that X-irradiation produces a uniform rise in both the fractions.

The data obtained with the *in vivo* glycogenesis suggest that the metabolic processes involved in the formation of glycogen from glucose are augmented as a result of irradiation exposure.

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STUDIES ON THE PATHOGENESIS OF ACUTE INFLAMMATION

I. CHANGES OF ENDOTHELIAL PERMEABILITY IN RABBIT EAR-CHAMBERS INJURED BY HEAT

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THE increased permeability induced by injury in vascular endothelium has been recognized as a key event in the evolution of acute inflammation. Escape of intravascular solute and of formed elements *via* altered endothelium has been held essential for a successful defence against noxious biological, chemical and physical agents. Despite the type of injury, increases of endothelial permeability were remarkably uniform qualitatively. The magnitude of permeability change was, however, apparently directly proportional to the intensity of trauma. As a result of many experimental studies it has been suggested that derangement of endothelial integrity during acute inflammation was causally related to material released from injured tissue ; substances considered as potential mediators of permeability alteration have been histamine, (Lewis and Grant, 1924), leukotaxine, (Menkin, 1955), certain polypeptides, (Duthie and Chain, 1939), 5-hydroxytryptamine, (Spector and Willoughby, 1957) and proteolytic enzymes found in plasma, (Miles and Wilhelm, 1955). Since study of such agents depended eventually upon an *in vivo* characterization by bioassay, variable behaviour in different species resulted in confusion as to the ultimate significance of each material in the genesis of increased vascular permeability. Additionally, different methods used for extraction and preparation of these substances caused uncertainty as to their actual existence in inflamed tissue. As a result, the exact biochemical mechanism responsible for endothelial alteration remains unknown.

Along somewhat different lines, study of experimental bacterial skin infections revealed vascular permeability to be increased maximally 1–6 hr. after lesions were established ; thereafter permeability returned toward pre-injury levels (Burke and Miles, 1958). Significant enhancement of infection in similar lesions was accomplished by one of several manoeuvres ; by inducing peripheral vascular collapse, by administering either adrenalin locally or Liquoid systemically 2–4 hr. after infection was initiated (Miles, Miles and Burke, 1957). Modification of peripheral blood flow in a like manner either before or after this critical time did not materially alter the evolution of infection. Since this decisive period coincided with the time when the exudative phenomena were most prominent, it was interpreted as evidence of the need for patent vascular channels to supply opsonins and phagocytic cells from circulating blood required for early control of infection. It was not apparent, however, why such infections were not modifiable either before or after this narrow time zone.

Previous microcirculatory studies have shown injured blood vessels to be highly permeable to plasma containing vital dyes. Although capillaries have been regarded as the major site of fluid loss during inflammation, venules were also believed to be a significant source for the leakage of plasma. There has been, however, no systematic microcirculatory study of acutely injured tissue designed to determiue quantitatively either the general pattern of plasma exudation from all segments of the peripheral circulation or the exact anatomical site of major losses of fluid.

The present studies were concerned with permeability changes of blood vessels in rabbit ear-chambers injured by heat. This inflammatory reaction has been described in detail previously (Allison, Smith and Wood, 1955) and proved an ideal model for study with Evans Blue Dye (henceforth referred to as T–1824) as the indicator for increased permeability of endothelium. It was found that major leakage of dye occurred in lesions 1–3 hr. of age and was less prominent immediately after injury and in older lesions. Stasis of blood flow with occlusion of highly permeable patent vessels was the event most obviously responsible for halting leakage of dye stained plasma. Greatest staining of tissue was observed at definite anatomical portions of the vasculature ; principally about thoroughfare channels or around capillary-venous radicals near arteriovenous fistulae.

METHODS

The type of ear-chamber, animals, basic experimental conditions and technique for producing tissue injury by heat have been described previously (Allison, Smith, and Wood, 1955). The inflammatory reaction produced by heat injury in the rabbit ear-chamber has been studied microscopically many times and served as the control for experiments described herein.

Five ml. of a 4·0 per cent aqueous solution of T-1824 (W761–1 lot no. 002093, generously supplied by Warner-Chilcott Laboratories Division, New York) was diluted in 15·0 ml. pyrogen free isotonic saline for intravenous administration. More dilute solutions of dye suitable for clinical use were not satisfactory because tissue staining was not distinct at high magnifications. This volume of dye solution was administered intravenously over a period of 3 min. *via* a suitable vein in the ear not being used for microscopic studies. The procedure was well tolerated initially but after 10–14 days, rabbits often developed paralysis of the hind legs and were killed.

Concentration of dye in plasma was determined from blood obtained at appropriate intervals from suitable ear veins. Usually 2·0 ml. of blood was drawn into syringes containing 0·1 molar sodium oxalate to give a final ratio of blood to anticoagulant of 9 : 1. After thorough mixing by repeated inversion, samples were centrifuged for 10 min. at 2000 rpm and plasma removed by gentle suction. No evidence of haemolysis was detected. Optical density readings of plasma containing dye were made with a Beckman model B spectrophotometer at 615 mμ and compared to control plasma diluted in similar fashion (Markus and Feigen, 1955). Plasma samples obtained during the first 24 hr. were diluted 1 : 500 in distilled water to obtain satisfactory spectrophotometer readings. After 24 hr., plasma samples were diluted 1 : 100 since dye concentration was much lower. Colour photographic records using 35 mm. film were made with equipment identical to that described previously (Allison, Smith and Wood, 1955).

RESULTS

Clearance of T-1824 from the Blood Stream

It is known that plasma concentrations of T-1824 are influenced by several factors among which are : (a) mixing during circulation ; (b) binding by plasma proteins, principally albumin ; (c) leakage or filtration into tissue with binding

EXPLANATION OF PLATES

Figs. 2, 3, 4, 5.—This series exemplifies leakage of T-1824 in ear-chambers of rabbits injured either immediately before or immediately after administration of dye. The tissue was burned 11 min. before dye was given in this experiment.

FIG. 2.—Approximately 15 min. after T-1824 was injected. Note major leakage of dye was from venous channels closely associated with arterial connections. The shadow (S) over lower half was cast by external portion of platinum pin.

FIG. 3.—Same ear-chamber as Fig. 2 but 1 hr. after dye administration. A broad ring of dye extended peripherally but not into avascular central zone (C).

FIG. 4.—Same as Figs. 2 and 3 but 6 hr. following injection of dye. Note clearing of T-1824 from peripheral portion of chamber but persistence of colour about platinum pin (C). Stasis of arteries (A) had begun and early perivascular haemorrhage was present.

FIG. 5.—Same as Figs. 2, 3 and 4 but 24 hr. after dye had been given. Tissues other than central portion (C) appear free of dye. Arteries noted previously (A) were completely static and surrounded by haemorrhage.

Figs. 6, 7, 8 and 9 depict intense staining observed when dye was given 1–3 hr. after thermal injury (compare with previous series). The ear-chamber was injured 2 hr. before was injected T-1824 in this series.

FIG. 6.—About 15 min. after dye was given. The intense ring of blue stain is obvious and has extended into central zone (C).

FIG. 7.—Same preparation as Fig. 6 but 1 hr. after giving dye. Blue colouration was greatly intensified and had begun to extend peripherally.

FIG. 8.—Continuation of Figs. 6 and 7 but 6 hr. after dye was given. Note almost complete clearing of tissues containing vessels.

FIG. 9.—Same series as Figs. 6, 7 and 8 but 24 hr. following T-1824 administration. The only visible dye was in the central zone (C).

Fig. 10, 11, 12 and 13 are representative of endothelial permeability found 5 or 6 hr. after heat injury. This particular preparation was burned 6 hr. prior to T-1824 administration.

FIG. 10.—Although slightly larger, little leakage of dye was noted in this lesion 15 min. after T-1824 had been given. Supernatant cells (SC) in upper right and lower half washed in from periphery after burn.

FIG. 11.—Same as Fig. 10 but 1 hr. after injection of dye. Note only traces of colour in contrast to that seen in Fig. 7.

FIG. 12.—Same as Fig. 10 and 11 but 6 hr. after loading with T-1824. Continuous leakage of dye resulted in a blue ring in the central avascular area (C) but little elsewhere. Dye was concentrated by supernatant phagocytic cells (SC).

FIG. 13.—Same as Fig. 10, 11 and 12 but 24 hr. after injection of dye. Peripheral tissues appeared clear of dye except where large numbers of supernatant phagocytes (SC) were present. Persistence of dye in the central zone (C) can be seen.

Fig. 14, 15,16 and 17 are representative of dye leakage in ear-chambers injured 24 hr. before T-1824 was given.

FIG. 14.—No staining was visible 15 min. after dye had been given. Haemorrhage arising from static vessels about the central platinum pin may be seen.

FIG. 15.—Same as Fig. 14 but 1 hr. after intravenous loading with dye. Again, no leakage into tissue was detected.

FIG. 16.—Same as Fig. 14 and 15 but 6 hr. after dye was given. Still no evidence of major escape of dye into tissue. Fresh haemorrhages (H) noted in upper half developed after animal struggled.

FIG. 17.—Same as Fig. 14, 15 and 16 but 24 hr. following injection of T-1824. For the first time, slight staining with dye was noted at lower left side of platinum pin.

FIG. 18.—T-1824 was given 3 hr. after heat injury. Perivascular staining occurred only about vessels (PV) and not about static channels (SV).

FIG. 19.—Same as Fig. 18 but higher magnification. Note lack of staining about static vessels (SV) as contrasted to intense colour adjacent to patent venules (PV). Blue colour of plasma in partially filled vessels (V) was due to plasma skimming.

FIG. 20.—Leakage of dye from artery (A) 2 hr. after injury and 10 min. after dye was given. This artery near central area (C) was subsequently occluded by static blood.

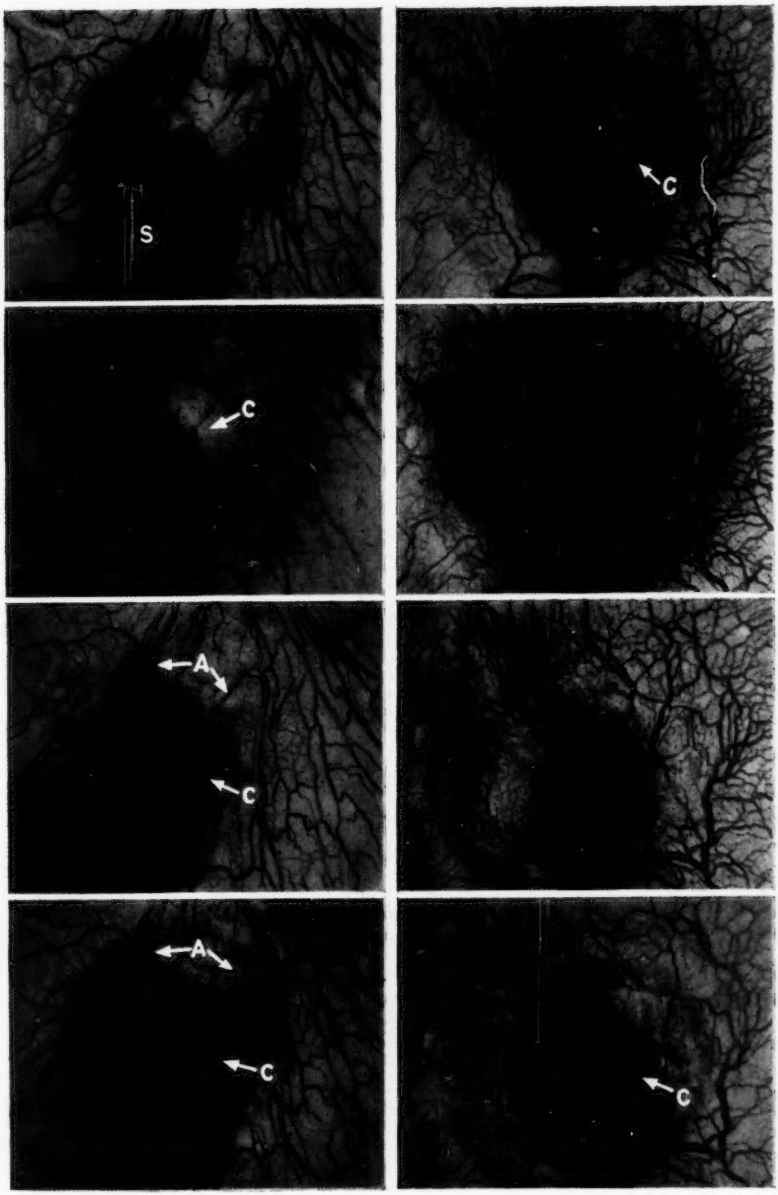
FIG. 21.—Concentration of T-1824 within granules of perivascular cells (PC) 24 hr. after injury and administration of dye. These cells, presumably macrophages, were next to wall of artery (A). Note shunting of flow caused by injury.

FIG. 22 and 23 illustrate that vessels most permeable after injury eventually became static. As in Fig. 22 taken 1 hr. after injury, two tributaries of a draining vein permitted a large quantity of dye to escape within 15 min. after dye had been given. The small static vessel (SV) extending into central zone did not allow extensive loss of dye. When Fig. 23 was taken 3 hr. after injury, one vein (V) on left was static and the other vein (V) on right eventually became occluded. In meantime, much dye migrated into central zone.

FIG. 24.—Two hours after T-1824 had been given 2 distinct rings of blue dye (1 and 2) were visible in an ear-chamber injured 3 hr. earlier. A portion of a third ring had been present on left side but was obscured by rapid spreading of dye.

FIG. 25.—Another example of ring phenomenon ; 1 hr. after T-1824 had been given and 3 hr. after injury. Note width of outer ring (1) as it spread to cover almost all of periphery. The inner ring (2) migrated in toward central avascular zone.

Fig. 22 and 23 were from Type A Kodachrome film ; all others were from Ektachrome film. Prints were made by Eastman Kodak Company as Kodachromes.



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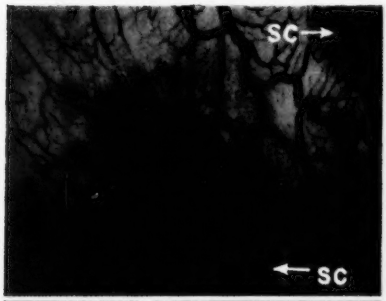

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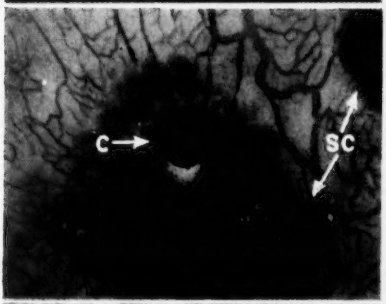

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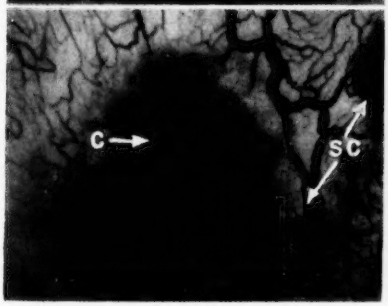

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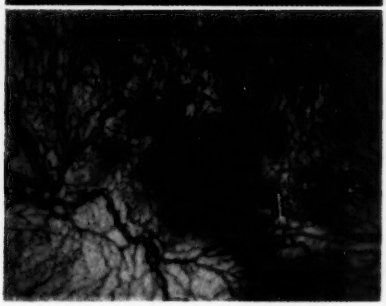

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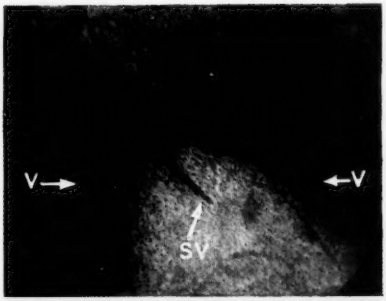

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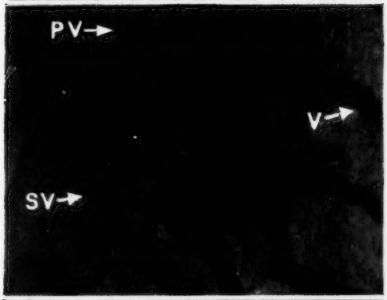

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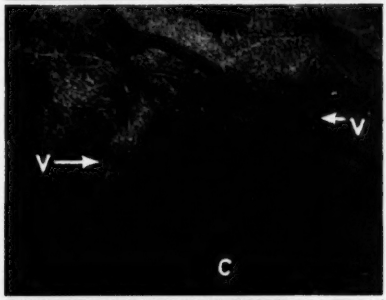

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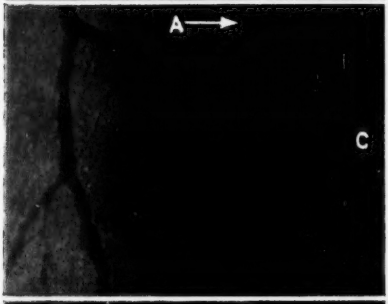

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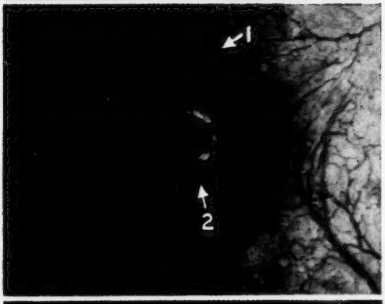

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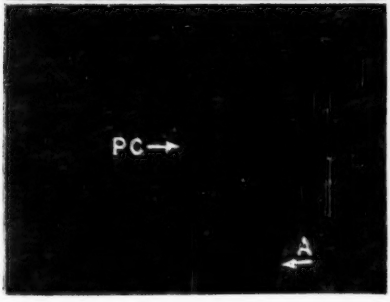

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by cells; and (d) recirculation *via* tissue lymph (Markus and Feigen, 1955). The half life for T-1824 has been determined for a number of species including rabbits but never at concentrations used in these experiments. Accordingly, it was necessary to determine persistence of dye intravascularly during the times microscopic studies were to be performed since the magnitude of leakage would be directly proportional to the plasma concentration.

Rabbits were bled for control samples and then given 20 ml. of 1 per cent solution of T-1824. Samples of venous blood were obtained 5 min., 1, 2, 3, 4, 5, 6, 24, 48, and 72 hr. after loading with dye. By the time administration of dye was completed, a deep blue colour was visible in the conjunctivae, nose, gums,

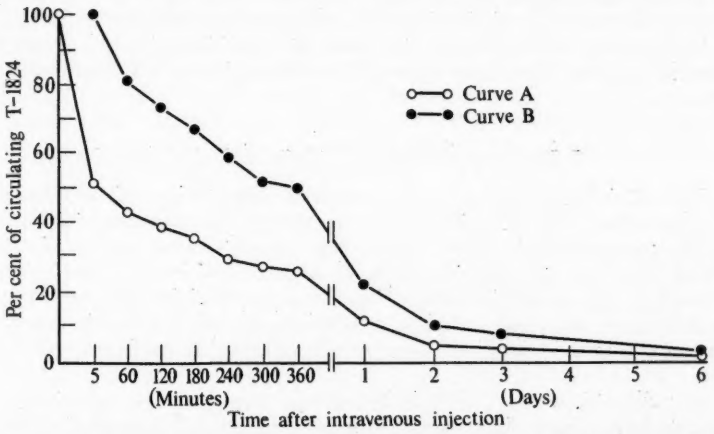

FIG. 1.—Mean disappearance curves of intravenously administered T-1824 in 4 rabbits. The first point on curve A, zero time, represents predicted plasma concentration; obtained by multiplying total quantity of dye administered (0·2 gm.) × total plasma volume (38 ml./kg. body weight). Other points on curve A were obtained by comparing actual concentration with 100 per cent value. The initial point on curve B, 5 min. after injection, was determined and assigned value of 100 per cent for comparison with all subsequent samples.

and ears. Little change in colour was seen after 10–14 days when most animals were killed; however, gradual loss of blue colour was observed in animals surviving 3 months or longer.

As may be seen in curve A of Fig. 1, concentration of dye in venous blood 5–11 min. after intravenous loading was only 50 per cent of anticipated levels based on calculations of total plasma volume of rabbits. Thereafter, plasma levels declined gradually over the ensuing week. The fact that plasma values were not as predicted probably was a result of rapid leakage before mixing was complete of unbound T-1824. Binding of this quantity of dye to plasma proteins required several minutes *in vitro* after thorough mixing. Thus it was conceivable that a large amount of free T-1824 did escape into tissue or was phagocytosed during the first minutes of mixing *in vivo*. Binding of dye by plasma proteins was complete *in vivo* 5 min. after loading when the first sample of blood was obtained since precipitation of protein with 5 per cent sodium tungstate completely decolourized the supernatant. When the value of the 5 min. specimen was used as a reference

point, a curve was obtained showing decline of available dye in circulation during times of microscopic study (Curve B, Fig. 1). From curve B, Fig. 1 it may be seen that only 50 per cent of dye was still in the circulation 6 hr. after loading. Preliminary electrophoretic studies indicated that saturation of albumin was not complete at concentrations of T-1824 used and no binding by globulins was detected.

From these results two facts were apparent. First, binding of dye by plasma insured identification of major changes of permeability induced by injury since rapid leakage of intravascular protein occurs during inflammation. Second, rapid clearance of dye from the circulation within a few hours after administration limited valid microscopic quantitation of tissue staining to a relatively short period. To obtain data of permeability changes throughout the first 24 hr. of acute inflammation it was necessary, therefore, to administer dye to rabbits with ear-chamber lesions of various ages. The following account is a description of these studies.

Behavior of T-1824 in Uninjured Rabbit Ear-chambers

No local staining of tissue about arterioles, capillaries and venules was seen in fully mature ear-chambers free of all evidence of inflammation. Vessels containing blood appeared dark bluish brown but vascular tone, rate and volume of blood flow were not altered. Vascular channels devoid of erythrocytes but filled with plasma appeared bright blue. The colour was less intense within 6 hr. and only a faint blue cast was visible in plasma 24 hr. after administration of dye. Vessels containing red blood cells regained their usual brownish red hue the following day.

Within 30 min. a faint diffuse blue tinge was noted in connective tissue. Lymph also had a light blue colour but in no instance did it equal the intense hue seen in plasma. Connective tissue of uninjured chambers never became intensely stained even when followed for 10–14 days. Within 24 hr., however, scattered cells located perivascularly, within lymphatics, and on the surface of ear-chamber tissue were intensely stained. The dye in these cells, presumably macrophages, was concentrated within cytoplasmic granules (Fig. 21). Persistence of T-1824 stained cells was noted for as long as 8 weeks after administration when observations were discontinued.

In no instance was there evidence of altered intravascular haemostasis since platelets, erythrocytes and leucocytes did not become adhesive or appear changed in number. Platelet thrombi were not encountered nor were conglomerations or sludges of red blood cells. Similarly there was no evidence of significant leucopenia or leucocytosis after administration of T-1824. Vascular tone, rate and volume of blood flow were not detectably modified by loading with dye. No effort was made to record blood pressure or temperature changes when T-1824 was given; however, lack of notable systemic response indicated little if any contamination with bacterial pyrogens.

Behavior of T-1824 in Injured Rabbit Ear-chambers

General pattern of tissue staining after thermal damage.—Since leakage of dye was unpredictable in thick ear-chambers composed of several layers of super-

imposed blood vessels, experiments were limited to thin preparations containing only one vascular stratum. Lesions under such circumstances were more consistent as to staining, size, and intensity of inflammatory response. Extravascular dye was most often seen in injured preparations as early as 3 min. after administration of T-1824 whereas in a few ear-chambers little or no staining was seen until 30 min. after injection. No T-1824 was noted about vessels filled with static blood at the outer rim of the lesion (Fig. 18, 19, 22). Staining of tissue developed only about blood vessels still patent at the periphery of injured tissue. It was further noted that tissue staining was most prominent either, about thoroughfare channels and arteriovenous shunts opened when other channels of blood flow were occluded, or along arterioles bordering on injured tissue (Fig. 20). Unless near an arteriovenous communication capillaries did not permit escape of dye as freely as vessels in which hydrostatic pressures were near that of the arterial circulation. In the next 3–6 hr. stasis of circulation extended peripherally to occlude highly permeable patent vessels. Thus, vessels permitting greatest escape of dye early in the reaction could be predicted almost with certainty to become static later (Fig. 22, 23). Sufficient dye to cause microscopic staining locally did not appear about remote vessels in mid-chamber and peripheral tissue.

As perivascular dye reached maximum concentrations, a narrow blue ring formed along the outer edge of injured tissue (Fig. 2, 6, 18). The inner margin of the blue ring slowly enveloped blood vessels within the zone of stasis whereas the outer edge lay over patent vessels (Fig. 7). Colouration of tissue was sharpest approximately 1 hr. after dye had been injected; thereafter the ring became wider, more diffuse and less intense (Fig. 4, 8). The quantity of stain appearing in extravascular tissue, as shall be discussed in subsequent sections, was directly related to the time that had elapsed between injury and administration of dye.

In an occassional preparation during the first hour after loading, T-1824 appeared to concentrate in distinct lines within several zones of the lesion (Fig. 24, 25). The concentric distribution of the lines as a series of rings imparted the appearance of a target to the ear-chamber. The first or outermost ring was peripheral to the static rim of the lesion and appeared to migrate away from the centre before becoming indistinct. The second or middle ring lay over the static zone and tended to migrate centrally to join dye in the third or inner ring. This last formation was immediately adjacent to the platinum pin and was the only part of the phenomenon that persisted past the second or third hour after dye administration. Staining of tissue between the lines of dye was less intense yet stain was obviously present in small quantity. Distribution of T-1824 in this fashion was transient since continuous migration obliterated the pattern.

Within 3 hr. after T-1824 had been administered, the blue ring began to fade as dye diffused both peripherally and through the static zone into the central ischaemic portion of the lesion (Fig. 4, 8). Escape of T-1824 from the centre, zone of greatest damage, was retarded since this area continued to be sharply stained 6 and 24 hr. later. All tissue of the ear chamber except the central zone appeared light blue 24 hr. after administration of dye (Fig. 5, 9, 13, 17). Phagocytic cells accumulated sufficient dye to stand out distinctly by this time (Fig. 12, 13, 21). Apparently the large number of these cells, in this instance predominantly leucocytes, accounted for persistence of dye about the platinum pin long after undamaged connective tissue was cleared of dye. Thereafter, little change in intensity of staining was observed within the next 7–14 days. Clearance of

dye *via* ear-chamber lymphatics was of minor significance in many preparations since these structures were rudimentary and often without anastomosis to the greater lymphatic system of the ear.

Tissue staining by T-1824 given immediately before or immediately after injury.—Little difference in staining intensity was noted when T-1824 was injected either immediately before or after ear-chamber tissue had been damaged by heat. As may be seen in Fig. 2, 3, 4, and 5 which are representative of ear-chambers studied shortly after injury, definite staining of perivascular tissue was present 15 min. after injection of dye (Fig. 2). One hour after administration (Fig. 3) slight intensification of colour developed and the width of dye-containing tissue was increased. After 6 hr. (Fig. 4) the discolouration was much less distinct at the periphery of the lesion but concentration in the central zone was definite. Little change was noted 24 hr. after injection although by this time tissue phagocytic cells contained granules deeply stained with blue dye (Fig. 5).

Tissue staining by T-1824 injected 2 hr. after injury.—Approximately 1–3 hr. after heat injury endothelial permeability was greatly enhanced. No sharp or abrupt increase occurred at this time since the change was gradual and varied slightly in each animal. For purposes of illustration, ear-chambers 2 hr. after damage were selected for documentation since this time fell within the period of maximum change. From Fig. 6, an ear-chamber injured 2 hr. earlier, it can be seen that a large amount of dye escaped into perivascular tissue 15 min. after T-1824 was given. One hour after injection of dye (Fig. 7) a bright blue ring had formed which was much more intense than when dye was administered immediately after injury (compare with Fig. 3). Within 3–6 hr. after dye was given, 5–8 hr. after injury, staining was diffuse with spread both centrally and peripherally (Fig. 8). The following day much fading had occurred with persistence of dye only in the region of the platinum pin (Fig. 9).

Tissue staining by T-1824 *injected 6 hr. after injury.*—Patent blood vessels located nearest to injured tissue began to be occluded by stasis 3 hr. after injury. The process was usually advanced by the sixth hour and accompanied by early perivascular haemorrhage. Thereafter, haemorrhage became much more intense about these obstructed blood vessels. A progressive fall in tissue staining by T-1824 was noted after inflammation had been in progress 3 hr. or longer regardless of the size of the lesion. Even though minor variations in diameter occurred, the same changes in tissue staining were noted even in exceptionally large burns. As an example, 15 min. after intravenous loading very little perivascular dye was seen in ear-chambers injured 6 hr. previously (Fig. 10) and colour intensity (Fig. 11, 12, 13) never did approach that seen when T-1824 was injected 2 hr. after injury (compare with Fig. 6, 7, 8, 9).

Tissue staining by T-1824 injected 24 hr. after injury.—Dye administered 24 hr. after thermal injury resulted in a staining pattern similar to that when T-1824 was given 6 hr. after heat damage. Dye escaped only about patent vessels and not in the vicinity of either empty vascular channels in the central zone or vessels filled with static blood in the lesion edge (Fig. 14, 15, 16, 17). Distribution of stain was usually spotty; notably about venous channels near arteriovenous shunts. Within 6–8 hr. after injection a small quantity of dye accumulated within phagocytic cells of the central zone and persisted throughout the remainder of observation. Little discolouration of peripheral tissue developed during the ensuing 24 hr. and a distinct ring of blue was never visible about the lesion.

Influence of T-1824 on other Aspects of Inflammation

Vascular tone.—There was no evidence intravenous loading with T-1824 caused significant alteration of either volume or rate of blood flow in thermally damaged ear-chambers of rabbits when compared with control. Vasodilatation accompanied by increased amplitude of arterial pulsations developed as expected; furthermore, dilatation of the circulation persisted through the 24 hr. period of observation.

Perivascular haemorrhage.—Extrusion of erythrocytes from static blood vessels was not impaired by T-1824 as may be seen in Fig. 4 which depicts early haemorrhage 6 hr. after injury and intravenous administration of dye. Likewise, there was no evidence T-1824 enhanced red blood cell diapedesis either by increasing vascular fragility or by depressing the blood clotting mechanism. Although not a prominent phenomenon, intravascular thrombi were as common as usual in injured ear chambers.

Intravascular formed elements.—Formation of rouleaux by erythrocytes was not prevented or accentuated by T-1824 and circulatory stasis with agglutination of red blood cells either singly or in clumps progressed in the usual manner. No coating of blue dye was seen on the surface of these sticky cells. Adhesiveness of platelets to injured endothelium and to marginated leucocytes was not altered by T-1824. As mentioned previously, platelets formed occlusive thrombi and mixed emboli as in unstained controls.

Sticking of white blood cells to segments of injured endothelium was not influenced by loading with Evans Blue Dye. Within 15–30 min. after injury unilateral sticking was the earliest evidence of leucocytic response to acute injury (Allison, Smith and Wood, 1955). The sticking reaction became progressively heavier and was fully developed within 1–2 hr. after trauma despite previous administration of dye. Between 6–9 hr. after thermal damage and administration of dye, sticking of white cells diminished in intensity. By 24 hr., only light sticking persisted despite vasodilatation otherwise consistent with acute inflammation. Diapedesis of white blood cells began shortly after sticking developed and was not impaired as to rate and intensity during the period of observation. Leucocytes gradually accumulated within the central zone of greatest injury eventuating in a dense infiltration 24 hr. after injury and administration of dye. Many such cells with intracytoplasmic dye stained granules remained near the platinum pin for many days and were responsible for persistence of colour in this region.

The formation of the peculiar intravascular globular structures after heat injury noted in previous studies was not influenced by T-1824 loading. It was also of interest that these globular bodies did not accumulate sufficient dye to stain blue.

Effect of T-1824 on Repair of Ear-chambers Injured by Heat

Repair of damaged tissue progressed gradually 2–3 weeks following acute insult. Restoration was chiefly distinguished by revascularization of injured tissue either by recanalization of static and collapsed vessels or by ingrowth of completely new endothelial buds; the latter event was less common. There was no evidence T-1824 impaired replacement of damaged connective tissue.

DISCUSSION

These microcirculatory studies demonstrated a distinct and relatively brief time after acute heat injury when endothelium was most permeable. This was in agreement with findings derived by others from study of inflammation produced by bacterial infections and chemical injury (Rigdon, 1940; Burke and Miles, 1958). During the 24 hr. after injury in our experiments, leakage of fluid continued at a reduced rate and, although not as dramatic as oedema formed after macroscopic burns, this late alteration of permeability was believed consistent with larger lesions.

Although no attempt was made to investigate the basic mechanism responsible for increased permeability, certain of our observations deserve comment since they bear directly on the experimental studies of other investigators. Exudation of fluid within the first few minutes after injury may have resulted from several events. First, any trauma could induce sufficient physical structural change to modify endothelial continuity and integrity; however, in these ear-chamber experiments mechanical disturbance was kept at a minimum. Nevertheless, distortion of tissue undoubtedly occurred and, although probably slight, must have caused some escape of dye. Second, liberation of histamine and/or serotonin from perivascular mast cells may have contributed to the immediate staining visible after injury. Histamine reputedly participates prominently in the early burst of vascular permeability after injury but its influence is transient and probably exhausted after 30–60 min. (Spector and Willoughby, 1957). Serotonin has also been suggested as responsible for enhancing endothelial permeability, particularly in rats (Rowley and Benditt, 1956), but in rabbits it had little effect on the integrity of blood vessels (Sparrow and Wilhelm, 1957). Third, the permeability promoting substances demonstrable in dilute plasma of several species and associated with β-globulins have also been found in rabbit plasma. Careful study revealed it to behave as an enzyme polypeptide that was antagonized by certain proteolytic enzyme inhibitors yet it was quite distinctly not endogenous plasmin (Wilhelm, Mill, Sparrow, Mackay, and Miles, 1958). Fourth, the degradation of cellular matter by the immediate surge of proteolytic activity from tissue injured by heat (Ungar and Damgaard, 1954) may result in other substances capable of modifying endothelial integrity. The precise importance of these various permeability promoting substances in the evolution of acute inflammation remains to be defined; yet in view of their biological activity, they most probably are of some significance. It is possible, therefore, that early increases of endothelial permeability noted in these experiments could have been caused by any one if not all of the factors discussed above.

The exaggerated leakage of T-1824 observed 1–3 hr. after heat injury suggested either gradual accumulation of material capable of increasing permeability of undamaged endothelium or progressive deterioration of injured endothelial cells. Selective antagonism by pharmacological agents has been accepted as strong circumstantial evidence for the existence of substances capable of increasing permeability within inflammed tissue. Problems related to their demonstration and quantitation have been reviewed recently by Spector (1958). Previous experimental data indicated that enzymatic breakdown of injured tissue released active substances, primarily peptides, capable of enhancing vascular permeability of recipient animals. Although peptides of various length were studied, short

chain structures were found to be most active (Spector, 1951) yet their participation in the pathogenesis of inflammation has not been exactly delineated. Leukotaxine, as described by Menkin (1955), is another substance of considerable biological activity yet of unknown significance with reference to late alterations of endothelium *in vivo*. In a similar way, the permeability promoting factors found in diluted plasma (Wilhelm, *et al.* 1958) have not been implicated precisely in the modification of endothelium that develops 1–3 hr. after injury.

An alternate explanation for the delayed increase of permeability need not entail participation of any of the biologically active materials discussed above. Rapid diffusion of dye through the lesions (Fig. 3, 7) suggested that the viscosity of injured ear-chamber tissue was quite low. In a fluid environment of this type it seemed unlikely that molecular substances smaller than plasma proteins such as short chain polypeptides had achieved high concentrations locally. If this were the case it would seem as reasonable to relate endothelial alteration to the original damage as to relate it to traces of material of questionable existence and activity. Thus it could be postulated further that as these damaged cells gradually deteriorated they slowly lost their functional integrity as a semipermeable membrane. Sticking of white blood cells and platelets, deposition of fibrin, and eventually circulatory stasis were visual proof of the extreme injury these segments of the vasculature had sustained. Since these latter events are generally associated with the blood clotting mechanism it did indicate in this instance at least the release of thromboplastic substances either from damaged endothelium or from perivascular connective tissue. Eventually, as the most permeable patent vessels were occluded by stasis, staining of connective tissue was sharply curtailed. It seemed probable therefore that obstruction of blood flow by stasis partially explained the reduced permeability noted in our ear-chamber experiments 3 hr. or more after injury. For example, when dye was administered 6 hr. or more after injury, stasis presumably prevented extensive staining of tissue since the most permeable vessels had already been occluded. Similar conclusions were reached by Sevitt (1949) in the study of larger burns with T-1824 used as an indicator of endothelial integrity.

It is now justifiable to compare these results with those obtained by Rigdon, (1940) and Burke and Miles (1958). First, their description of a period of greatest permeability induced by both chemical and bacterial trauma coincided with our finding of a similar time of greatest leakage of dye in ear-chambers damaged by heat. Disappearance of the enhanced permeability phase in ear-chambers could be correlated with occlusion of previously patent porous blood vessels. Stasis of blood flow was a consistent feature of such ear-chambers injured by heat and appeared responsible for arresting excessive exudation of intravascular T-1824. In the skin infections studies by Burke and Miles (1958) stasis of blood flow was not believed responsible for decreasing leakage of dye after 4 hr. had elapsed but this particular phase of the reaction was not looked for. It is highly probable that stasis did occur commonly in their studies, especially in infections resulting in tissue necrosis. since cessation of blood flow always accompanies macroscopic death of tissue. That this was so in their type of experiment will require additional documentation. Their experiments resembled ours, however, in that infection or injury was limited as to magnitude and duration and was not self perpetuating with progressive involvement of tissue. It is thus tempting to conclude that any injury sufficiently intense to produce death and necrosis of

tissue should result in permeability changes of the type described in this paper, whereas less intense injury, *i.e.* not causing death of cells, should not cause the same sequence of events since occlusion of circulation by stasis is not an expected event. As expected after milder degrees of trauma, dye escaped into tissue for a brief time shortly after injury and in much smaller quantities. Significant late seepage 2–6 hr. after injury was not expected and did not occur.

A second point worthy of comment was the obvious relation between enhancement of experimental bacterial infections by modifying local blood flow (Miles, Miles, Burke, 1957) and the time when endothelium was most permeable. Reduction of blood flow about infected tissue during a critical time amounted to an anatomical blockade of the lesion with protection of noxious agents from blood borne elements of the host defence. As a result bacteria could then proliferate with little restriction until the circulation was re-established. Control of infection was thus delayed until blood flow returned ; the result was damage to a greater area of tissue. If on the other hand circulation in the vicinity of the lesion was not restricted until 4 hr. after inception of infection, no enhancement of disease occurred. It was thus apparent that not only had something already happened locally which enabled the host to control the infection but that this something responsible for containment was related to the previous circulatory adequacy. From our studies it is apparent that considerable exudation occurred before the sixth hour and that stasis of blood flow with vascular occlusion was the prime limiting factor of the reaction. Thus, any event limiting flow of blood during this critical period of high permeability will seriously compromise the ability of the host to contain infectious agents.

After Landis (1927) showed a direct relation between stasis and rapid leakage of fluid from injured blood vessels. it was reasoned that increased permeability was the major cause of stasis. The finding in our experiments that little dye leaked from vessels found to be static immediately after injury was therefore of interest (Fig. 2). Presumably, the time of greatest loss of fluid was so short that too little escaped to be detected even though T-1824 had been given before injury. This type of stasis developed only in channels immediately occluded by injury and although totally devoid of active blood flow were in communication with patent channels. Of additional note was the fact that the agglutinated erythrocytes did not adhere to endothelium. Stasis developing later in the reaction, however, was accompanied by considerable leakage of fluid and there was a distinct tendency for the agglutinated erythrocytes to stick to endothelium. Major escape of fluid continued only so long as movement of the blood continued and occasionally this covered a span of several hours. Obstruction of previously patent channels in this instance was apparently related to change of cellular surfaces since development of stasis was usually associated with intense sticking of the formed elements to each other and to endothelium. This type of stasis differed from that seen immediately after injury since interruption of flow in patent channels occurred only after a pronounced change of all cellular membranes. It, like leucocytic sticking, may possibly be related to a phase of blood clotting.

Without fail, it was the patent vessel bordering heat injured tissue that was the major site for exudation of intravascular solute (Fig. 22). In most instances these vessels were venules but less intense leakage of dye was noted about capillaries and arterioles (Fig. 20). Staining of tissue was most prominent about those portions of the vasculature receiving arterial blood *via* shunts opened after

injury had occluded normal channels of flow. Segments of vessel bordering the lesion but some distance from arterial connections permitted much less dye to escape. The importance of intravascular hydrostatic pressures in the exudation of fluid as emphasized by Landis (1927) was thus confirmed. It is understandable therefore why leakage of fluid from capillaries was not prominent since they did not commonly participate in forming arterial shunts. In no instance was there evidence of a permeability gradient along various segments of vessels as described by Rous and Smith (1931). The thickness of arterial walls seemed to limit escape of dye although marked perivascular staining was noted when an artery was extensively injured (Fig. 20).

Differential staining of connective tissue with vital dyes absorbed to plasma proteins as depicted in Fig. 24, 25 has not been reported previously. While studying the circulatory phenomena of experimental macroscopic burns in guinea pigs, Sevitt (1949) encountered the formation of a single ring of blue after T-1824 had been injected. Secondary rings were not recorded although in less severely burned animals rapid migration of dye into the centre of the lesion was noted. In our experiments this curious target configuration observed 1–2 hr. after administration of dye was a transient event since the lines fused or faded after 15–30 min. No explanation was immediately available but several possibilities warrant consideration. Separations of the lines suggested migration of different plasma proteins labeled with T-1824 as might occur in an electrophoretic system. It is known that T-1824 migrates primarily with plasma albumin although if present in high enough concentration movement with the α-, β-, and γ-globulins can occur (Rawson, 1943). The electromotive force or injury potential required to exert a directional influence on movement of extravascular plasma proteins labeled with dye conceivably could exist between injured and normal ear-chamber tissue. Actually the conditions that obtain within both normal and injured ear-chambers as to pH, oxygen and carbon dioxide tensions are not known although previous studies (Allison, Sleator, and Wood, unpublished) revealed marked changes of electrical potential during vasoconstriction between 2 platinum electrodes installed within uninjured ear-chambers. Efforts to demonstrate a current of injury after thermal damage in such ear-chambers, however, were never successful due to insoluble problems of design. Nonetheless an electrical field induced by injury might modify the behaviour of plasma proteins in damaged tissue and result in the production of our ring phenomenon.

Another explanation of the rings may be one of diffusion of plasma proteins through the colloid of connective tissue and ground substance in a manner analagous to a chromatographic column. Ordinarily, as in other chromatography systems, the molecular configuration and size of the various proteins would influence the rapidity of movement. Thus one would not expect such rapid movement of proteins as seen in our ear-chambers but changes of tissue pH and lowered viscosity of ground substance could influence rates of movement in the direction we recorded. Otherwise, the lines could represent simple diffusion or flow of protein molecules through a semiliquid medium.

As a third and less likely explanation one might liken the blue circles to the precipitation of inorganic salts in certain colloidal systems as described by Liesegang, whereby a series of permanent lines or rings may be formed. The rings observed in our studies were evanescent and did not resemble the deposit of insoluble salts. Although an explanation was not available for this curious finding,

it is an interesting possibility that plasma proteins may in fact diffuse or migrate through injured tissue at different rates. Such a phenomenon might be of real physiological importance in instances where ground substance has been altered by disease. As an example an abnormally viscous ground substance could materially impede transport of plasma protein fractions needed for preservation of host defences, essential for metabolic equilibrium or perhaps required for repair of damaged structures. Similarly, abnormal plasma proteins might not migrate as expected in normal ground substance and thus not participate effectively in preserving homeostasis within the host's tissues.

SUMMARY

In these studies, rabbit ear-chambers injured by heat proved a highly satisfactory model for investigating changes of endothelial permeability demonstrable *in vivo* by intravenously administered T-1824. The following deductions could be made from our findings :

Injury by heat caused an immediate but modest increase of vascular permeability. A gradual but transient secondary increase of permeability ensued over the next 3–4 hr. but slowly subsided by the sixth hour. Thereafter, a slight increase of permeability persisted through the subsequent 24 hr. The possible significance of these findings was discussed.

Leakage of dye stained plasma was noted from all visible segments of the vasculature but was most prominent about vessels near an arterial communication. This association was believed to substantiate the important role of intravascular hydrostatic pressure in the transendothelial transport of fluid. For this reason, venous channels, the commonest site for arteriovenous shunts, were found to afford greatest loss of fluid.

Although stasis of blood flow was the event most prominently associated with enhanced vascular permeability, it could not be causally connected to the phenomenon. For example, vessels that became static immediately after injury were not responsible for major leaks of fluid. Yet, channels most permeable to T-1824 almost invariably became static although there was no set time for this to occur since some highly porous injured vessels remained patent for 4–6 hr. whereas others were occluded within 2–3 hr. It was thus concluded that enhanced endothelial permeability was not the lone factor responsible for stasis.

An unexpected finding was the remarkable blue rings that formed in 1–2 hr. after T-1824 administration about burns 2–3 hr. old. Although a precise explanation for their occurrence was not available, it was thought they might represent a distribution peculiarity of extravascular plasma proteins labeled with T-1824 under conditions of acute injury.

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THE EFFECT OF EPI-OESTRADIOL BENZOATE ON CHOLESTEROL INDUCED ATHEROSCLEROSIS IN THE CHICKEN

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OESTROGENS are reported to lower lipid levels in human subjects (Eilert, 1949; Barr, 1953) and to inhibit and reverse the lesions of experimental atherosclerosis in chicks (Pick, Stamler, Rodbard and Katz, 1952 *a* and *b*). They are not generally prescribed in the treatment of human atherosclerosis because feminisation of male subjects and other undesirable side-effects accompany their use. However, oestrogenic effects and anti-atherosclerotic action may be separable by chemical manipulation. This study is one of a series designed to assess the therapeutic value of certain semi-synthetic oestrogens. The substance under test in this experiment was epi-oestradiol benzoate.

METHODS

Seventy two Golden Legbar-Light Sussex cockerels, one month old, were allocated to 6 groups of 12 birds and housed in individual cages. Weight was distributed comparably in each group. Each group was subjected to an experimental regime of 16 weeks duration except one (group A) which was killed after 8 weeks to demonstrate the establishment of lesions before treatment. The birds were weighed at weekly intervals. The groups were as follows:

(A) Fed an atherogenic diet consisting of commercial chick mash supplemented by 1 per cent cholesterol for 8 weeks. (B) Fed the atherogenic diet for 16 weeks. (C) Diet as above for 16 weeks. After the 8th week they were given epi-oestradiol, 1 mg. intramuscularly daily. (D) Diet as above for 16 weeks. After the 8th week they were given epi-oestradiol, 5 mg. intramuscularly daily. (E) Commercial mash only for 16 weeks. (F) Commercial mash for 16 weeks. After the 8th week they were given epi-oestradiol, 1 mg. intramuscularly daily.

Blood for lipid analyses was withdrawn from an alar vein at the start of the experiment and at 4-weekly intervals thereafter. Total serum cholesterol was estimated by a modification of the Sperry-Schoenheimer technique (1934) and lipid phosphorus by the method of King (1951). The cholesterol-phospholipid ratio was calculated from these figures. Total phospholipid was estimated as 25 times the lipid phosphorus value. Lipoproteins were analysed by paper electrophoresis, Sudan black B staining, densitometry and planimetry as previously described (Watson, 1957). Complete sequential lipoprotein analyses were carried out only in groups B, D, E and F. Group C was included after the 8th week.

All birds except those in group A were killed at the end of the 16th week. The aorta, brachio-cephalic and iliac arteries were inspected and the degree of atherosclerosis graded as slight, moderate or severe. (*Slight* atheroma—focal lesions up to 2 mm. in diameter, of white or cream colour and accompanied by minimal thickening of the intima. *Moderate*—plaques up to 5 mm. in diameter, cream or ivory-yellow in colour with intimal thickening and longitudinal ridging. *Severe*—large plaques of cream to yellow colour merging into diffuse atheroma).

The hearts were fixed in 10 per cent formol saline and three transverse slices taken through mainly ventricular areas, excluding the extreme apex. One frozen section was prepared from each slice and stained by Sudan IV and haemalum. A microscopical count of atheroma in the coronary arteries was then carried out. Any sudanophilic intimal thickening or plaque was regarded as positive. Sudan staining without evidence of structural abnormality was regarded as negative. The incidence of atheroma was expressed as a percentage.

The condition of comb, wattles and testes was noted.

RESULTS

Weight

The monthly weight averages are shown in Table I. During the first 8 weeks there is no difference between the birds fed cholesterol free and cholesterol enriched mash. During the next 8 weeks, however; the cholesterol fed birds weigh on average 172 g. less than the others. We were unable to keep accurate records of food intake but our impression was that the lighter birds ate less. The cholesterol fed birds receiving oestrogen (C and D) were heavier than those receiving cholesterol alone (B).

TABLE I.—*Monthly Average Weights of Each Group (g.)*

Weeks	A	B	C	D	E	F
0	305	307	310	305	307	310
4	651	642	662	641	628	638
8	1280	1187	1138	1243	1288	1262
12	—	1580	1565	1527	1731	1755
16	—	1777	1851	1861	1973	2001

Oestrogenic effects

All birds receiving 5 mg. epi-oestradiol/day (D) showed marked hypoplasia of combs, wattles and testes. Seven birds in group C and 3 in group F showed similar changes. Three of the birds in group B showed partial inhibition of sex characters, but none showed changes as severe as in the oestrogenized animals. Feminising effects must therefore be considered a prominent feature of this oestrogen.

Cholesterol, phospholipid and C/P ratio

Serum cholesterol and phospholipid levels rose following the administration of the cholesterol enriched diet, the peak increase occurring at the end of the first month (Table II). Thereafter they fell, phospholipid reaching normal levels by the 3rd month, while cholesterol levels were still twice normal at the end of the 4th month. Epi-oestradiol, 1 mg./day, given to cholesterol fed birds had no effect on this pattern of cholesterol response, but did retard the fall in phospholipid so that the C/P ratio reached lower levels (group C). However, the 5 mg. dose of epi-oestradiol (group D) led to gross elevation of cholesterol and phospholipid levels, the latter to a greater degree, so that the C/P ratio was lower than normal.

The drug in 1 mg. doses produced a slight rise in phospholipid in those birds receiving normal mash (group F), but cholesterol was unaffected, so that here also the C/P ratio fell below normal.

TABLE II.—*Mean Monthly Lipid Values of all Experimental Groups*

	Group A			Group B				Group C			
	Cholesterol*	Phospholipid*	C/P ratio	Cholesterol*	Phospholipid*	C/P ratio	β/α lipoprotein	Cholesterol*	Phospholipid*	C/P ratio	β/α lipoprotein
Weeks											
0	189	9·9	19·1	209	9·5	21·9	0·61	188	8·9	21·1	—
4	955	14·7	65·0	1028	15·2	67·7	2·96	773	13·5	57·2	—
8	804	11·6	69·3	931	13·1	71·0	3·06	781	12·5	62·5	—
12	—	—	—	426	8·1	52·7	2·18	440	11·8	37·3	2·26
16	—	—	—	299	6·2	48·2	1·67	289	8·1	35·6	1·90

	Group D				Group E				Group F			
	Cholesterol*	Phospholipid*	C/P ratio	β/α lipoprotein	Cholesterol*	Phospholipid*	C/P ratio	β/α lipoprotein	Cholesterol*	Phospholipid*	C/P ratio	β/α lipoprotein
Weeks												
0	193	9·2	21·0	0·63	205	9·1	22·5	0·61	180	9·5	18·9	0·59
4	650	11·5	56·5	3·11	145	9·7	15·0	0·59	158	10·3	15·3	0·94
8	576	10·8	57·7	2·53	178	9·9	18·0	0·73	196	11·4	17·2	0·77
12	2513	159	15·8	—	185	8·6	21·5	0·91	175	10·6	16·5	1·70
16	3032	172	17·6	—	164	7·3	22·4	0·69	177	11·0	16·1	1·83

For reasons which are explained in the text, the β/α lipoprotein ratios of Group D, at the end of the 3rd and 4th months, were not calculable, but were obviously immense.

* (mg. per cent)

Lipoproteins

Figure 1 shows the lipoprotein pattern of a normal bird at the start of the experiment. This pattern was maintained in the normal control birds (group E). After one month of cholesterol feeding a new peak emerged and the pattern now resembled that of the adult human. Although the avian protein fraction involved in this lipoprotein complex is not identical with human β-globulin, the lipoprotein is virtually identical with human β-lipoprotein, as far as electrophoretic properties are concerned. The second peak coincides with the albumin/α_1-globulin band of chicken serum and is considered to be α-lipoprotein.

In group B the β/α lipoprotein ratio reaches maximum values in the 1st and 2nd months of the experiment and falls thereafter (Table II). This is due to the reduction in β-lipoprotein. The β/α ratio of group C corresponds to the group B values during the 3rd and 4th months.

Following the administration of 5 mg. epi-oestradiol the serum became grossly lipaemic and only fractional amounts (0·02 ml. instead of 0·10 ml.) were electrophoresed. With this smaller quantity no α-lipoprotein was visible, β-lipoprotein stained intensely and a third band appeared running ahead of β-lipoprotein and quite separate from it. This corresponds with α_2-globulin, which is also increased (Fig. 2). This figure also shows a band which corresponds to the pre-β-lipoprotein described by Besterman (1957).

In group F 1 mg. epi-oestradiol produced a rise in β-lipoprotein to levels about twice those in the normal control birds.

Aortic lesions

Only one of the cholesterol fed birds had a normal aorta (Table III). There was no obvious difference between the nature and severity of lesions in the treated and untreated groups. Two of the birds in group E had moderate lesions. This confirms the occurrence of atherosclerosis in otherwise healthy birds.

TABLE III.—*Incidence and Severity of Lesions in Coronary Arteries and Aortas of all Experimental Groups*

Group	Aortic lesions				Coronary lesions		
	Severe	Moderate	Slight	Absent	Number of birds with lesions	Total affected arteries	Total normal arteries
A	—	7	5	—	—	—	—
B	3	8	1	—	6	53	531
C	1	9	2	—	8	37	469
D	3	8	—	1	6	20	462
E	—	2	—	10	—	—	—
F	—	2	—	10	—	—	—

Coronary arteries in Group A were not examined.

Coronary artery lesions

The hearts of the birds in group A were not sectioned. No affected arteries were seen in the hearts of birds fed normal diets (E and F). The incidence of coronary lesions in the remaining experimental groups (B, C and D) is shown in Table III; analysis of variance reveals no significant difference between these groups ($F = 0{\cdot}97$). This finding is at variance with the report of Pick *et al.* (1952*a*) that oestradiol, a potent oestrogen, was effective in reversing the coronary artery lesions in cholesterol fed cockerels.

DISCUSSION

One approach to the prevention or treatment of atherosclerosis depends on the belief that it can be affected by control of serum lipids. As yet no wholly acceptable régime has been found. While some oestrogens have been shown to prevent or reverse lesions in experimental animals, none has been free of undesirable side effects. Epi-oestradiol, the oestrogen under test in this study, does not differ from other analogues in this respect, and no therapeutic claim can be made for it. It undoubtedly causes profound alterations in the circulating lipids but these are not associated with improvement in aortic or coronary lesions. In addition the degree of inhibition of sex characters is an adverse factor.

It is unlikely that any substance which increases the concentration of serum lipids can advance the therapy of atherosclerosis, unless the important factor is not so much the amount of lipid, as the balance between cholesterol and phospholipid (the C/P ratio), the main lipids involved. This experiment shows that gross increases in β-lipoprotein, cholesterol and phospholipid can occur while the C/P ratio falls below normal values.

Alpha-lipoprotein is little or not at all involved in these changes. Its apparent disappearance from the later group D lipoprotein patterns is probably due to the smaller amount of serum used for electrophoresis. The significance of the emergent α_2-lipoprotein is not clear.

SUMMARY

Epi-oestradiol benzoate has been tested in the treatment of experimental chicken atherosclerosis. Even after large doses (5 mg./day for 2 months) which produced total inhibition of the sex characters there was no improvement in either coronary or aortic lesions.

Some aspects of the associated lipid changes are briefly discussed.

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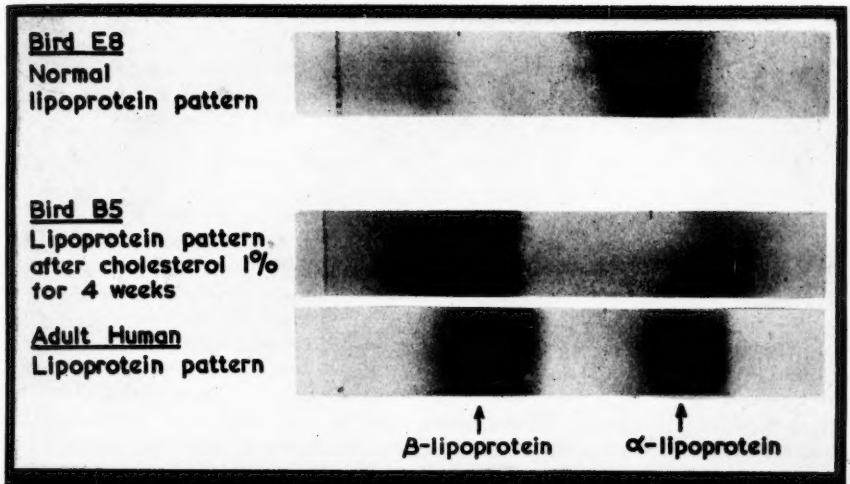
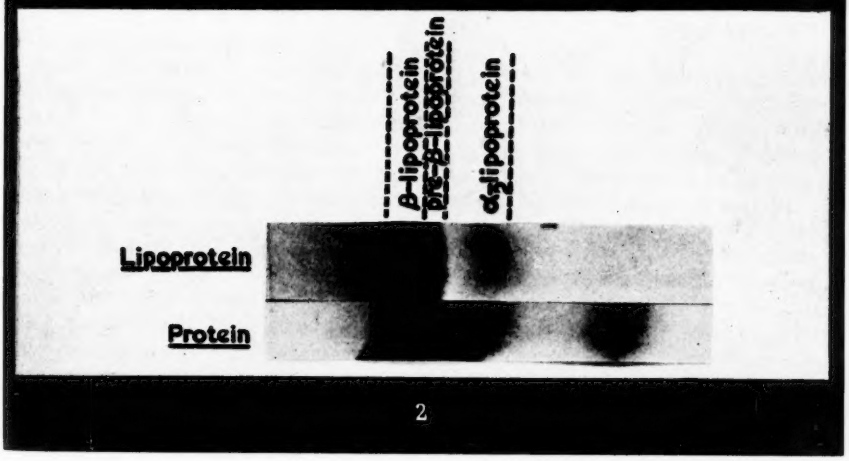
EXPLANATION OF PLATE.

FIG, 1.—Lipoprotein patterns of normal and cholesterol fed chicks.

FIG. 2.—Electrophoretic appearance of proteinsand lipoproteins after 5 mg. epi-oestradiol for 8 weeks (Bird D 12) 0·02 ml. serum.


Bird E8
Normal
lipoprotein pattern
Bird B5
Lipoprotein pattern
after cholesterol 1%
for 4 weeks
Adult Human
Lipoprotein pattern
β-lipoprotein
α-lipoprotein

1


β-lipoprotein
α2-lipoprotein
Lipoprotein
Protein

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THE QUANTITATIVE FLOCCULATION REACTION OF EQUINE TETANUS ANTITOXIN

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In 1951 a variety of partially purified, concentrated tetanus toxoids were produced in these laboratories for use in combined immunizing agents for public health purposes. Concentrated equine antitoxins, some pepsin treated, others only heat treated, were also produced. This afforded the opportunity to study the quantitative flocculation reaction in the tetanus system with a variety of reagents having different properties. Some 26 curves were prepared, involving 21 different pairs of reactants embracing 7 toxoids and 11 antitoxins. At the time the results were only briefly analyzed and were not published.

Recently a re-examination of the experimental protocols was undertaken in the hope that they might contribute to the important controversial question of the significance of the quantitative precipitation curves and their characteristics in the flocculation reactions of horse antibody. These curves and a mathematical-statistical analysis of them which contributes useful evidence on this matter are presented here. We have been unable to find previous publication of quantitative curves in the tetanus system.

Briefly, the issue is whether the linear portion of the flocculation curves (Pappenheimer and Robinson, 1937), derived from the diphtheria-toxin-horse-antitoxin system, represented a true zone of equivalence as the authors claimed, or was merely an artefact of other interfering precipitating systems. Further at issue is Pappenheimer's deduction that the slope of these lines measured the immunochemical purity of the antigen in terms of the fraction specifically precipitable. These questions, among others, were raised by Pope and his collaborators in a series of papers (Pope, Stevens, Caspary and Fenton 1951, Pope and Stevens 1953, Pope 1954 and 1957), and replied to by Pappenheimer and Yoneda (1957). Pope in turn replied to the latter paper in the same issue of the journal (Pope 1957). Reviewers in 1954 and 1955 appeared to have accepted the views of Pope (Oakley 1954, Marrack, 1955).

Although direct deductions as to the diphtheria system cannot be made from work on the tetanus system, there are obvious advantages to an intersystem comparison of similar procedures, especially where these systems belong to a unique group involving the antibodies of equine pseudoglobulin in reaction with their respective antigens.

MATERIALS AND METHODS

Reactants.—The properties of the reactants are given in Table I. In the tables and text the letter " A " stands for " antitoxin " and precedes their lot numbers. Corresponding use is made of the letter " T " for " toxoid." The pepsin treated antitoxins were processed by

TABLE I.—*Properties of the Antitoxins and Toxoids*

1 Designation	2 Pepsin used	3 AU per ml.	4 Lf per ml.	5 AU per Lf	6 mg N per ml.	7 Lf per mg. N	8 $\bar{a}$
A19L	−	1350	2000	0·68	20·4	100	0·06
21	−	2250	2500	0·90	26·8	90	0·10
11	+	1600	1700	0·94	14·2	120	0·13
19	−	2500	4700	0·53	26·1	180	0·13
60	+	2900	3600	0·80	19·2	190	0·13
24	−	1250	1750	0·71	29·0	60	0·14
62	+	4500	6000	0·75	30·6	200	0·18
57	+	3200	3600	0·89	21·1	170	0·28
52	+	4500	4500	1·00	20·3	220	0·31
58	+	5000	6000	0·83	19·7	300	0·32
59	+	5000	5000	1·00	20·8	240	0·35
							$\bar{b}$
T45	..	..	200	..	0·885	230	0·14
40	..	..	450	..	0·956	470	0·24
49	..	..	300	..	0·533	560	0·28
48	..	..	350	..	0·568	620	0·29
41	..	..	600	..	1·195	500	0·34
50	..	..	650	..	0·850	760	0·52
47	..	..	450	..	0·525	860	0·77

various modifications of Pope's method involving brief exposure to pepsin under conditions relatively unfavourable to rapid reaction. The 4 non-peptic antitoxins were heated and collected in the 0·3–0·5 saturated ammonium sulphate fraction, except A19L which was a low-salt (0–0·3 sat.) fraction. The toxoids were processed by a simple 2-stage ammonium sulphate fractionation (Levine and Stone, 1951). Nitrogen values of all reactants represent protein N, defined as nitrogenous substance non-diffusible through dialysing membrane. Since processing always involved exhaustive dialysis against water and saline, a total N assay, using the Markham modification of the micro-Kjeldahl method, (Markham, 1942) gave the required value.

Lf titration.—The Ramon optimum for both toxoids and antitoxins was determined in the conventional way, using constant antigen and varying antibody. The prime standard was an antitoxin obtained from the National Institutes of Health. It had an *in vivo-in vitro* ratio of 1·0. It was standardized against a stabilized laboratory toxin which was used to titrate our antitoxins. One of the latter, with a ratio close to 1·0, was the working standard against which the toxoids were titrated. False flocculating zones have rarely been encountered in our work and where present, have had their combining proportions remote from those of the specific zone. The evidence for the recorded Lf values for antitoxins representing the specific zone rather than some other precipitating system was as follows : The week to week changes in serum titres of the producing horses were carefully followed, and the yields in Lf units from native plasma to processed concentrate were uniform. The *in vivo-in vitro* ratios varied within a narrow range. These ratios are given for the antitoxins of the present study in Table I where it may be seen they varied from 0·53 to 1·00, mean 0·82. The evidence for toxoids was similar. Crude culture filtrates ranged in titre from 20 to 40 Lf per ml. Processing yields were uniform. Both crude and purified toxoids used in tetanus prophylactics at 5 Lf per dose, passed antigenicity tests at the expected level. All 7 of the toxoids of this study obtained such *in vivo* verification of their *in vitro* titres.

Reactions.—Twenty-six reactions were carried out, 5 of which were duplicates. The reacting pairs are listed in Table II. For brevity a reacting pair will be designated without letters, the toxoid being listed first. Thus "Pair 41–21" means "the reaction T41 plus A21." Eighteen of the reactions were carried out with 600 Lf units of antitoxin in a final volume of 5 or 6 ml., corresponding to a concentration of 100 or 120 Lf per ml. Others were done at concentrations between 70 and 240 Lf/ml. In some cases incomplete curves were completed by a second reaction, sometimes at a different concentration. Seven curves remained incomplete, but yielded useful data. Lf values were used as guides to the desired

combining proportions, and to establish the location of the Ramon optimum point in the curve. The graphs, however, were based exclusively on weight units. The procedure was to add the antitoxin, always contained in 1 ml. volume, to conical micro-Kjeldahl digestion tubes. Saline was added to give the final volume, allowance being made for the increasing increments of toxoid, in duplicate or triplicate for each increment, which was the final addition. The tubes were incubated for 3 hr. at 37° and then placed at 5° overnight. The floccules were washed twice with cold saline, using the centrifuge for separations, and were assayed for N by the micro-Kjeldahl method. Graphs were made by plotting mg. toxoid N (TN) added per mg. antitoxin N (AN), against mg. ppt. N found per mg. AN. This method of plotting placed all curves in comparable scale regardless of reaction concentration, or of variations in the amount of specific AN used from experiment to experiment.

RESULTS

The curves of 15 of the 21 reaction pairs are presented in Figs. 1–3. In general these curves resembled those obtained in the diphtheria system by Pappenheimer and Robinson (1937), Cohn and Pappenheimer (1949) and Bowen, Polley and

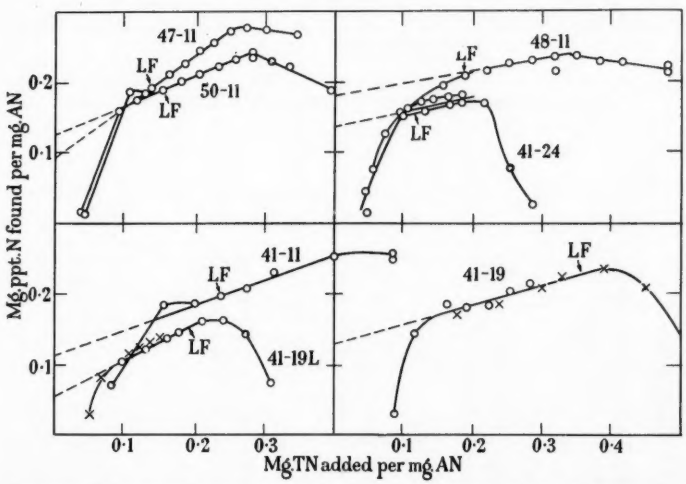

FIG. 1.—Quantitative precipitation curves for 7 tetanus toxoid-equine antitoxin reactions involving different reacting pairs. Each pair is represented by a hyphenated number, the first of which designated the toxoid lot, and the second the antitoxin. The point at which the Lf of each reactant was identical is indicated. All curves have a definite linear portion, involving not fewer than 5 experimental points, invariably including the Ramon optimum. In the case of 3 curves, the left and right hand section were run as 2 successive experiments. In 2 cases the common centre sections were in the same line, indicating reproducibility. In the case of Pair 41-24, the centre sections were parallel but slightly displaced.

TN = Toxoid nitrogen ; AN = Antitoxin nitrogen. (Referring to the protein N of the whole reagent used in each case.)

Huang, (1954). Despite distortions in some cases, which may have been due to other precipitating systems, a line could in every case be drawn which sloped upward to the right, passing through or near not less than 4 and upwards of 10 experimental points. The Ramon flocculation optimum was invariably in this line. The remainder of the curve always fell away from the line at its extremities,

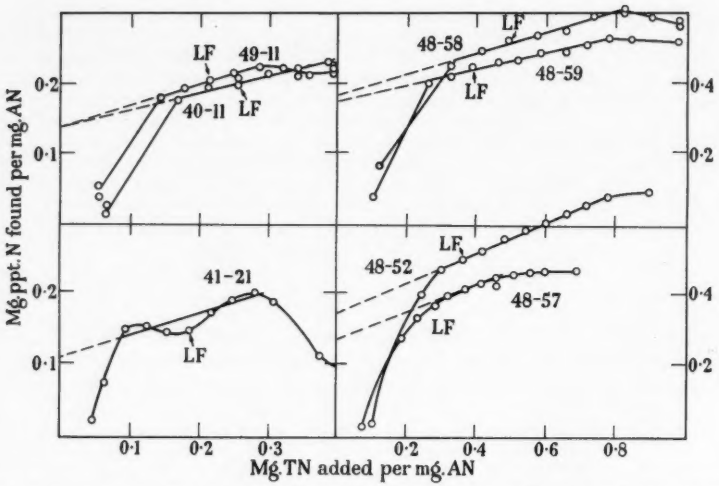

FIG. 2.—See caption for Fig. 1. Note, however, that the scale of both co-ordinates had to be increased to accommodate the 4 curves on the right.

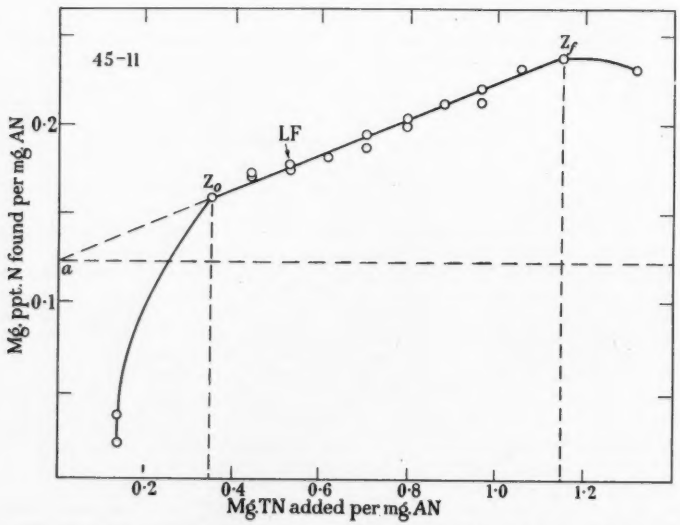

FIG. 3.—The quantitative precipitation curve for the reaction between T45 and A 11. Experimental points are shown in duplicate except where the N assay check was so close as to represent a graphical distance less than the radius of the indicating circle. This rule for the most part was followed in Figs 1 and 2 also. Z_o and Z_f indicate the beginning and end, respectively, of the linear portion. Dotted lines are constructs for geometrical analysis.
TN = Toxoid nitrogen; AN = antitoxin nitrogen. (Referring to the protein N of the whole reagent used in each case.)

except in the case of 7 incomplete curves (not shown). With respect to 4 of these, sufficient tubes had not been carried into the region of antigen excess, so that the terminal points of their lines were not defined. This circumstance showed the unexpected variations in lengths of these lines, the significance of which will appear later. These lines were otherwise well defined and yielded useful data. The remaining 3 of the 7 curves were incomplete with respect to both beginning and end of the lines, but the slopes and intercepts were established by not less than 4 points including the Ramon point, and verified by comparison with complete curves representing duplicate runs with the same reactant pairs.

It seemed natural to take a mathematical interest in these lines, not only in their slopes and intercepts, but also in 2 other variables which completed their definitive parameters, namely the coordinates of their extremities (Z_0 and Z_f, Fig. 3). The latter were obvious variables because there were great differences in total length and in locations of the lines in the graph. Table II lists the intercepts (a) and slopes (b) respectively, for all reacting pairs including duplicates, obtained from the equation of the line, calculated in most cases by the method of least squares using the experimental points that were considered to define

TABLE II.—*Line Parameters of* 21 *Pairs of Reactants*

					6	7
					Abcissae of	
1 Experiment No.	2 Pairs T–A	3 Intercept a	4 Slope b	5 a/b	Start x_0	Finish x_f
2	41–21	0·10	0·47	0·29	0·09	0·30
3+4	41–21	0·11	0·29	0·29	0·09	0·30
5	41–11	0·13	0·29	0·38	0·13	0·40
6	41–11	0·13	0·34	0·38	0·13	0·37
7+10	41–19	0·13	0·27	0·38	0·16	0·38
8+11	41–19L	0·06	0·48	0·18	0·09	0·21
9–12	41–24	0·14	0·22	0·41	0·10	0·21
13	40–11	0·14	0·24	0·54	0·18	0·38
14	47–11	0·10	0·70	0·17	0·09	0·25
25B	47–11	0·09	0·73	0·17	<0·14	>0·22
16	48–11	0·18	0·16	0·45	0·20	0·36
25C	48–11	0·16	0·26	0·45	<0·20	>0·30
18	45–11	0·12	0·10	0·93	0·35	1·15
25A	45–11	0·13	0·11	0·93	<0·53	>0·82
19	49–11	0·14	0·28	0·46	0·18	0·28
20	48–58	0·37	0·30	1·10	0·36	0·83
21	48–59	0·35	0·23	1·21	0·28	0·80
22	48–52	0·34	0·43	1·07	0·30	0·78
23	48–57	0·28	0·36	0·96	0·28	0·47
24	50–11	0·13	0·40	0·25	0·08	0·29
26D	45–52	0·31	0·20	2·21	0·92	>1·49
26E	47–52	0·29	0·78	0·40	0·19	>0·40
26F	50–52	0·31	0·65	0·60	0·22	>0·45
27+28A	47–58	0·26	0·62	0·42	0·29	>0·55
27+28B	47–60	0·14	0·96	0·17	0·22	0·48
27+28C	47–62	0·18	0·87	0·23	0·21	0·38
	Total	4·19 (21).	9·02 (21).	12·81 (21).	4·92 (21)	7·93 (17)
	Mean	0·200	0·430	0·610	0·234	0·466

Five pairs were in duplicate.
Indefinite values were due to incomplete curves ; see text.

the line. In a few cases the graphical method was used to draw the line and to read off the values for a and b.

Hypotheses that these slopes and intercepts were specific properties or functions of particular reactants were set up and tested by an analysis of variance (Mather 1946) of these parameters within and between toxoids and antitoxins for all 26 reactions involved in the study. The results of this analysis are given in Table III which also lists the 4 hypotheses considered, the conclusions made,

TABLE III.—*Variance Analysis of Intercepts and Slopes within and Between Toxoids and Antitoxins for* 26 *Reactions, of which* 5 *were Duplicates*

	Intercept, a			Slope, b		
Variance component	Sum of squares	DF	Mean square	Sum of squares	DF	Mean square
Total, corrected	0·2162	25	..	1·4454	25	..
Between A's	0·2026	10	0·0203	0·7339	10	0·0734
Within A's (duplicates in)	0·0136	15	..	0·7115	15	0·0474
Within duplicates	0·0004	5	0·0001	0·0256	5	0·0051
Within A's (duplicates out)	0·0132	10	0·0013	0·6859	10	0·0686
Between T's	0·0963	6	0·0160	1·2268	6	0·2045
Within T's (duplicates in)	0·1199	19	0·0058	0·2186	19	0·0115
Within duplicates	0·0004	5	0·0001	0·0256	5	0·0051
Within T's (duplicates out)	0·1195	14	0·0085	0·1930	14	0·0138

Hypothesis	Variance ratio, F	DF	P	Result
a is a function of A's	0·0203/0·0013 = 15·6	10/10	$\ll$0·001	Accepted
a is a function of T's	0·0160/0·0085 = 1·88	6/14	0·1–0·2	Rejected
b is a function of A's	0·0734/0·0686 = 1·07	10/10	$>$0·2	Rejected
b is a function of T's	0·2045/0·0138 = 14·8	6/14	$\ll$0·001	Accepted

and the confidence levels thereof. The first hypothesis considered was that the values of the intercepts were properties of the antitoxins. This hypothesis was accepted because the variance of the intercepts between the different antitoxins was 15·6 times as great as their variance within each antitoxin. The probability of this occurring by chance is considerably less than 0·001 (Fisher and Yates, 1949). Fig. 4 is a graphic demonstration of the validity of this conclusion. For purposes of clarity, only 2 antitoxins are considered. Each has been reacted with a variety of toxoids, designated by number, the linear portions only of each curve having been drawn as solid lines, extensions to the ordinal intercept being broken lines. The lines fall into 2 distinct groups, the lower group representing A11 and the upper group A52. It is obvious from this figure how the variance of the intercepts between the 2 antitoxins greatly exceeds that within either one of them. It seems likely that the variance within each group represents experimental error.

The second hypothesis tested was that the values of the intercepts were properties of the antigens. The variance ratio was low, the probability due to chance high, and therefore the hypothesis had to be rejected.

The third hypothesis, that the values of the slopes were determined by the antitoxins, had to be rejected on the same grounds as the preceding.

Finally, the fourth hypothesis, that the values of the slopes were properties of the toxoids, demands acceptance inasmuch as the variance of slopes between toxoids exceeded that within each toxoid by 14·8 fold, with a confidence level of the same order as that of the first hypothesis. In analogy with Fig. 4, Fig. 5 demonstrates the latter finding visually. The linear portions of the curves of a variety of antitoxins reacted with 2 toxoids, T47 (dashed extensions) and T48 (dotted extensions), have been drawn as solid lines. Note that the intercepts, unlike the case of Fig. 4, appear randomly distributed along the ordinate, but that

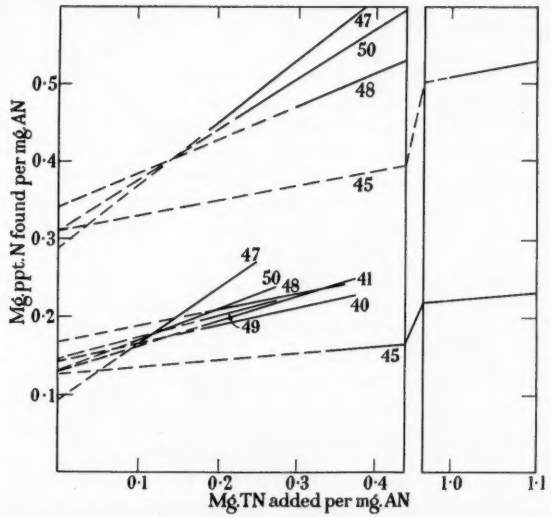

FIG. 4.—Linear portions of the curves of 2 antitoxins, A 11 (lower group) and A52 (upper group), reacted with a variety of toxoids, designated by number. Dotted portions are extrapolations to show intercepts. This is a visual demonstration of the significance of Table III. Note how the variance of intercepts between the two antitoxins greatly exceeds their variance within either one of them. At the same time, note that the slopes of the toxoids are with one exception, in the rank order of their specific activities, as listed in Table I. Compare with Fig. 5.

TN = Toxoid nitrogen; AN = antitoxin nitrogen. (Referring to the protein N of the whole reagent used in each case.)

the slopes clearly fall into 2 distinct groups, the higher slopes all having dashed extensions (T47), the lower slopes dotted extensions (T48). It is obvious from this graph that the variance of the slopes between the 2 toxoids greatly exceeds the variance within them.

The overall conclusion of the foregoing analysis is that the probability that the slopes and intercepts are real properties of the toxoids and antitoxins, respectively, considerably exceeds 1000 to 1. On the basis of this evidence, the mean values of a and b, designated $\bar{a}$ and $\bar{b}$, averaged over all available estimates, and therefore considered, in each case, to be the best estimate of the true value, have been incorporated in Table I as intrinsic properties of the reactants. In all subsequent analysis, these mean values will be used in preference to the values for each individual experiment.

The next step in the analysis was to consider what property of the reactants these parameters represented. Two independent lines of evidence will be presented that indicate that slope and intercept of toxoid and antitoxin, respectively, directly measure the immunochemical purity of these reagents, where the latter term is defined as the weight fraction of the reagent that is specifically precipitable at equivalence.

Reference to Fig. 4 and Table I will disclose 2 interesting facts not previously mentioned. Firstly, the antitoxin with the higher average intercept, A52, also has the higher specific activity, 220 Lf per mg. N, against only 120 for A 11.

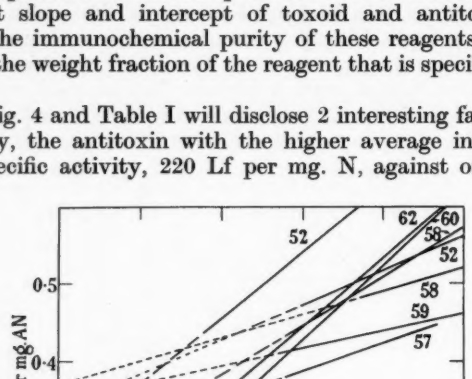

FIG. 5.—Linear portions of the curves of 2 toxoids, T47, dashed extensions, and T48, dotted extensions, each reacted with a variety of antitoxins, designated by number. Note that T47, having the higher specific activity of 860 Lf per mg. N, has a uniformly higher slope than T48, whose activity is only 620. Note also that the intercepts of these lines tend to follow the rank order of specific activity of the antitoxins as listed in Table I. Compare with Fig. 4. This also demonstrated visually the significance of Table III, as it is clear that the variance of slopes between T47 and T48 greatly exceeds the variance within either one of them.

TN = Toxoid nitrogen; AN = antitoxin nitrogen. (Referring to the protein N of the whole reagent used in each case.)

Secondly, and more significantly, the slopes of the various toxoids reacted with these antitoxins, which at first glance might seem random, are not random but with one exception are in the rank order of their specific activities. Likewise in Fig. 5, the toxoid having the high slopes, T47, also has the higher activity, 860 Lf per mg. N, as against 620 for T48. And the intercepts of the various antitoxins that were reacted with these toxoids are not randomly distributed, but closely follow the rank order of their specific activites. In order to test the relationship which the above observations suggested using the information available from the whole of the data, the antitoxins and toxoids were arranged in Table I in the increasing rank order of their intercepts and slopes respectively, and their corresponding specific activities are listed. The correlation coefficients between $\bar{a}$ and $\bar{b}$ on the one hand, and the specific activities on the other, were calculated and

found to be 0·78 ($P < 0·01$) in the case of antitoxins, and 0·901 ($P < 0·01$) in the case of toxoids. These high correlations and their high confidence levels, involving specific activity, which may be regarded as a measure of "relative purity," constitute the first item of evidence for slope and intercept measuring the purity of toxoid and antitoxin. It should be remarked here that the antitoxin correlation is inferior to that of the toxoid, probably because the overall purity range of the antitoxins was much smaller than that of the toxoids (The purest antitoxin available was only 0·35 specifically precipitable), and the molecular weight changes due to peptic cleavage theoretically increases the specific activity, without affecting the immunochemical purity, thus tending to distort the correlation between these variates.

The second source of evidence stemmed from a consideration of the positioning and delimiting of these lines in the graph. We have designated the beginning and end of a line, that is, the point at which the curve falls away from the line at its extremities (Fig. 3), as Z_0 and Z_f and their coordinates as x_0, y_0 and x_f, y_f. In Table II, Columns 6 and 7, we have listed only the abscissal coordinates of these points as read directly from the graphs, the ordinates being immediately available from the equation of the line which, of course, is $y = bx + a$.

In considering the factors that might influence the various points along the abscissa at which the linear portion of the curves commenced for different reacting pairs, it seemed apparent that, say, 1 mg. of a relatively pure antitoxin, with its higher proportion of specific N, would require more toxoid (higher x_0), than a less pure antitoxin, At the same time, the toxoid would have an effect, namely, more of a less pure toxoid (also higher x_0) would be required, than of a more pure toxoid. Another way of looking at this is to say that the beginning of the line will be displaced to the right if the antitoxin is more pure and the toxoid less pure ; it will be displaced to the left if the reverse is true. It thus appeared possible to test the hypothesis that $\bar{a}$ and $\bar{b}$ represent the purity of antitoxin and toxoid, respectively, by examining the requisite that x_0 shall be proportional directly to $\bar{a}$ and inversely to $\bar{b}$, that is, by examining the relation

$$x_0 = k\frac{\bar{a}}{\bar{b}}. \tag{1}$$

The values of $\bar{a}/\bar{b}$ are listed in Column 5, Table II. Note that these are not necessarily the ratios of Column 3 to Column 4, since, as was indicated above, the mean values are the best available estimates of the true values. Columns 5 and 6 now contain 26 pairs of values of the variables whose relationship it is desired to examine. Fig. 6 is a graph of the 21 independent pairs of these values, the mean value of x_0 for the 5 duplicate curves having been used. The least squares line for the regression of $\bar{a}/\bar{b}$ on x_0 was drawn. The regression variance greatly exceeded all other variance and indicated significance at $P < 0·001$. The correlation coefficient between x_0 and $\bar{a}/\bar{b}$ was 0·906. The equation of the regression was

$$x_0 = 0·40\frac{\bar{a}}{\bar{b}} - 0·0084 \tag{2}$$

The intercept, — 0·0084, represents a narrow miss of the origin, as can be seen from Fig. 6, and is small for all real values of the other term. Therefore it is

justifiable to attribute it to experimental error, in which case we have the simple relation

$$x_0 = 0{\cdot}40\,\frac{\bar{a}}{\bar{b}}. \tag{3}$$

This function and its high significance constitute the second item of evidence in favor of the hypothesis set up above, namely, that the intercept and slope of the linear portions of the flocculation curves measure the immunochemical purity of antibody and antigen respectively.

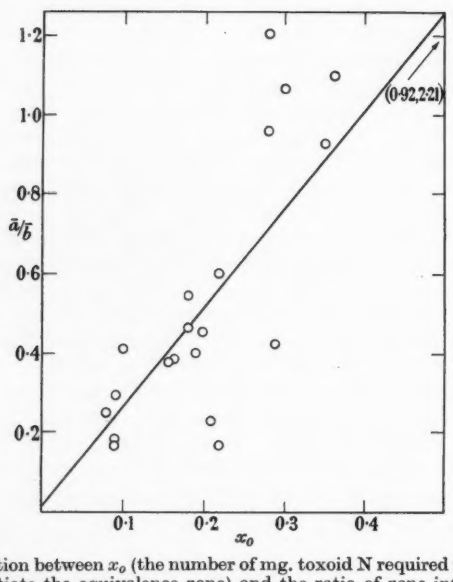

FIG. 6.—The correlation between x_0 (the number of mg. toxoid N required to be added to 1 mg. antitoxin N to initiate the equivalence zone) and the ratio of zone intercept to slope, for 21 different reacting pairs. The correlation coefficient was 0·906, $P < 0{\cdot}001$.

Analogous reasoning will anticipate that x_f, the terminal point of the line, should also be a linear function of the ratio of the purity of the reactants, that is, of $\bar{a}/\bar{b}$. This regression was similarly solved and was found significant at $P < 0{\cdot}001$. The correlation coefficient between x_f and $\bar{a}/\bar{b}$ was 0·804, somewhat less than in the case of x_0. Two factors may account for this. One is that only 17 pairs of observations were available due to the 4 incomplete curves in which the termini were not defined. The other is the evidence of interfering systems in the high antigen region, indicated by the failure of many curves to drop downward vertically, but rather to trail off almost horizontally. This phenomenon was carefully analyzed by Cohn and Pappenheimer (1949) with rabbit serum. The effect would be to increase the error of x_f.

The equation of this regression was

$$x_f = 0{\cdot}91\,\frac{\bar{a}}{\bar{b}} - 0{\cdot}024 \tag{4}$$

The graph was quite similar to Fig. 6 and therefore will be omitted. The intercept, — 0·024, represents, as in the previous case, a narrow miss of the origin, and can be dropped. We now have the relation

$$x_f = 0{\cdot}91\,\frac{\bar{a}}{\bar{b}}. \tag{5}$$

which, together with (3), makes available the coordinates of the extremities of the linear portions of the flocculation curves of theoretical reaction pairs having any assigned immunochemical purities. These coordinates are as follows :

$$x_0 = 0{\cdot}40\,\frac{a}{b},\ y_0 = a + b\,x_0 \tag{6}$$

$$x_f = 0{\cdot}91\,\frac{a}{b},\ y_f = a + b\,x_f \tag{7}$$

The lines of Fig. 7 were drawn using these coordinates and the indicated arbitrarily selected values of a and b, representing reacting pairs of various purities. From all the evidence adduced above, it is felt that Fig. 7 represents theoretical families of curves which correspond to the completely defined equivalence zones of the tetanus toxoid-equine antitoxin reaction when that reaction is not distorted by interfering systems. The line nearest the upper left hand corner is of special interest as it represents the theoretical equivalence zone for absolutely pure reactants. Zones for the reactions of the present study would fall only in the lowest third of the graph because none of the antitoxins exceeded 0·35 in purity. The goodness of fit between the experimental and theoretical lines is measured on the average by the magnitudes of the above correlation coefficients upon which the derivations depend. Figs. 4 and 5 show how the zone lines vary in length, and shift horizontally and vertically in accordance with the above theoretical requirements.

DISCUSSION

It is difficult to understand how the foregoing mathematical-statistical relationships could have been demonstrated for the linear portions of the curves presented in this study, unless these lines represented true equivalence zones. Acceptance of these lines as zones of equivalence requires, by definition, that any residual reactants found in the supernatants, either free or as complexes, and any non-specific protein N that may contaminate the floccules are merely contributions to error variance and can be ignored in the case of experiments in which the error component is relatively small. The latter may be estimated from the variance due to curvature of the zone line, if significant ; otherwise, from the variance of experimental points about the line of least squares. The direct proof of equivalence in this zone by supernatant analysis, radioactive tracer methods, etc. have been inconclusive and have been the subject of debate for about a decade. The evidence presented here for the tetanus system has been of an entirely different character.

The justification for entering evidence obtained in the tetanus system into discussion of a controversy centering in the diphtheria system is that the contributors to this field, including the reviewers, have generalized the findings to the unique flocculation reaction of horse antibody with certain (but not all)

categories of antigens. The exotoxins are among these categories. The evidence of the present study further justifies the generalization.

Pope *et al.* (1951) said : " In a very large number of experiments which we have carried out ... we have failed to obtain a single curve of the type reported

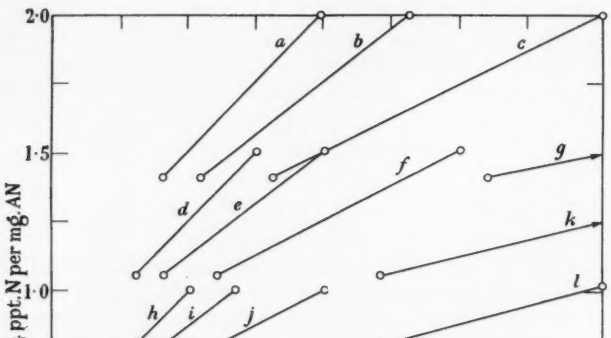

FIG. 7.—Theoretical equivalence zones for reacting pairs of tetanus toxoids and antitoxins of arbitrarily selected levels of immunochemical purity. The line nearest the upper left corner corresponds to reagents of absolute purity. a = the purity of the antitoxin and is the ordinal intercept of the line. b = the purity of the toxoid and is the slope of the line. TN = Toxoid nitrogen ; AN = antitoxin nitrogen. (Referring to the protein N of the whole reagent in each case.)

(a) $a = 1{\cdot}0$, $b = 1{\cdot}0$	(b) $a = 1{\cdot}0$, $b = 0{\cdot}75$
(c) $a = 1{\cdot}0$, $b = 0{\cdot}5$	(d) $a = 0{\cdot}75$, $b = 1{\cdot}0$
(e) $a = 0{\cdot}75$, $b = 0{\cdot}75$	(f) $a = 0{\cdot}75$, $b = 0{\cdot}5$
(g) $a = 1{\cdot}0$, $b = 0{\cdot}25$	(h) $a = 0{\cdot}5$, $b = 1{\cdot}0$
(i) $a = 0{\cdot}5$, $b = 0{\cdot}75$	(j) $a = 0{\cdot}5$, $b = 0{\cdot}5$
(k) $a = 0{\cdot}75$, $b = 0{\cdot}25$	(l) $a = 0{\cdot}5$, $b = 0{\cdot}25$
(m) $a = 0{\cdot}25$, $b = 1{\cdot}0$	(n) $a = 0{\cdot}25$, $b = 0{\cdot}75$
(o) $a = 0{\cdot}25$, $b = 0{\cdot}5$	(p) $a = 0{\cdot}25$, $b = 0{\cdot}25$
(q) $a = 0{\cdot}1$, $b = 1{\cdot}0$	(r) $a = 0{\cdot}1$, $b = 0{\cdot}5$
(s) $a = 0{\cdot}1$, $b = 0{\cdot}25$	(t) $a = 0{\cdot}1$, $b = 0{\cdot}1$

by Pappenheimer and Robinson (1937) or Cohn and Pappenheimer (1949) for horse antitoxin." In 1953 Pope and Stevens reported the tendency to form a sharp equivalence point instead of a broad equivalence zone as a sample of antitoxin was increasingly freed of other antibodies. The conclusion from this was that in general linear equivalence zones do not form, but that where lines are found they must be considered as artefacts of interfering non-specific precipitating systems.

The parameters of such lines could therefore have no immunological significance whatever.

The present study in the tetanus system reports reactions between 21 different pairs of reagents every one of which yielded curves of the classical shape to a greater or lesser degree, and in any case, adequate to define an unambiguous line for which great immunological significance has been demonstrated. Bowen *et al.* (1954), working in this laboratory, reported 34 curves in the diphtheria system, each a product of a different pair of reactants, for which unambiguous lines could be drawn. The great majority of these curves were perfectly classical in shape. Dr. Bowen has kindly made available the detailed protocols from which the following calculations have been made: The correlation coefficient between the specific activity of 13 diphtheria antitoxins and their zone intercepts, when each was reacted against a single toxoid, was 0·93, $P < 0{\cdot}001$. Eleven of these antitoxins, run against a toxin, yielded a coefficient of 0·925, $P < 0{\cdot}001$. One antitoxin, Lot 425, was run against 10 different crude toxins. The mean value of the intercepts of these 10 zone lines, measuring the immunochemical purity of this antitoxin, exceeded the standard deviation 18·2 fold, thus indicating the experimental constancy required by theory.

These high correlations between intercept and specific activity for different antitoxins, and the virtually constant intercept for the same antitoxin reacted with different toxins, could not have been demonstrated if the original concepts of Pappenheimer and Robinson were not valid.

The present data embrace a sufficient number of curves to establish that distortions from the ideal form, which are numerous, are not necessarily a property of one or another of the reactants, but rather of the particular pair itself. For when either member of the pair is reacted with a different partner, no distortions at all, or very different ones, may appear. Several examples of this may be seen in Figs. 1 to 3. In the reaction 41–11 (Fig. 1) there was a hump at the beginning of the line. The same toxoid in reactions 41–19L and 41–24 revealed no such hump. The same antitoxin, A 11, used in 47–11 (Fig. 1) and 45–11 (Fig. 3), and others, did not show the hump. The distortions of particular pairs, however, were characteristic and reproducible. Furthermore, the likelihood of distortions does not appear to be related to the purity of the reactants involved. Consider pair 45–11 (Fig. 3), selected as illustrative example in detailed analysis because of the close approach of its curve to classical configuration. The toxoid, T45, had the lowest specific activity of all seven, and the antitoxin, A 11, was only 4th among eleven in this respect. The quality of the curve apparently depends upon whether or not the optimal combining proportions of specific and major non-specific systems coincide. To the extent that they coincide in the case of a given pair, to that extent will the line be distorted, the precipitates contaminated with non-specific N, and the greater will be the error of any deductions made from the data. Most students of the quantitative precipitin methods appear to have considered that these methods are best employed and best validated when the purest obtainable reagents are used. The present study was designed on the opposite assumption, that the maximum information could be obtained from reagents covering a wide spectrum of relative purities. Only the latter design could have permitted the emergence of the correlations which validate the method and give estimates of its inherent errors. The greatest weakness of this study, in our opinion, was the use of antitoxins of varying, and largely unknown, degrees

of enzyme degredation, for this detracted materially from the correlation between specific activity and zone intercept. In a given series, the antibody should either be uncleaved native molecules, or molecules of uniform cleavage.

Pope (1953) considered that the demonstration of a crystalline diphtheria toxin of 3200 Lf per mg. N has invalidated Pappenheimer's quantitative flocculation method which indicated that pure toxin contained only 2200 Lf per mg. N. An even greater discrepancy of a parallel nature in the tetanus system emerges from the present study. The mean value of the specific activity corresponding to a slope of 1·0 available from the seven toxoids employed may be calculated from Table 1 to be about 1680 Lf per mg. N. This is less than half the value reported by Pillemer, *et al.* (1948) for crystalline tetanus toxin, namely, 3400 to 3600 Lf per mg. N. The precise explanation for these discrepancies is unavailable on the basis of present knowledge, but the following suggestions may be offered. The effort to obtain ever purer toxins has involved increasing manipulation of these labile proteins which carried with it the possibility of change in their molecular size without necessarily affecting their biological indicator reactions. Thus a constant immunochemical purity could be consistent with a varying specific activity. In the case of the crystalline toxin of Pillemer, it would appear that the Lf values served more to gauge the progress of purification than to establish an absolute characterization on a par with the other properties reported. No statement is made as to the prime standard antitoxin upon which the reported Lf values were based. The effort to correlate Lf values with the toxicity data is rendered difficult by reason of the fact that the Pillemer group measured mouse MLD's whereas most other workers have used guinea pig MLD's. That the Lf value in the tetanus system is difficult to carry over unchanged between different places and times is attested by the absence of an international standard flocculating serum. It is an advantage of the quantitative flocculation method that all data are on a weight basis and that purity of a reactant is estimated as the weight per cent specifically precipitable, rather than in terms of the uncertain and relative Lf unit per mg. N.

Part of the evidence in this study represents experimental fulfillment of theoretical requirements. Two previous studies which performed the same function may be mentioned in conclusion.

Cohn and Pappenheimer (1949) have shown that toxins evaluated with horse antitoxin by the zone method gave the same results as when evaluated with antibodies of other species according to the theoretical equations of Heidelberger and Kendall (1935).

The findings of Pappenheimer and Robinson (1937) in the diphtheria system gave good agreement with Goldberg's theory based on a statistical analysis of particle collisions. (Marrach, 1955 ; Goldberg, 1952).

SUMMARY

Quantitative flocculation curves for reactions between 21 different pairs of partially purified tetanus toxoids and antitoxins were prepared. Altogether 7 toxoids and 11 antitoxins of varying degrees of purity were involved.

Despite frequent distortions, possibly due to interfering precipitating systems, it was evident that the main tendency was to form linear zones within a limited range of combining proportions.

The 4 definitive parameters of the zones were measured, namely, slope, intercept, and coordinates of the beginning and end of the zone. Inter-relationships of these parameters were investigated.

The slopes of the zone lines were found to be highly correlated with the specific activities (Lf per mg. N) of the toxoids.

The intercepts of the zone lines were found to correlate significantly with the specific activities of the antitoxins.

It was postulated that if the slopes and intercepts were measures of the immunochemical purity of toxoid and antitoxin, respectively, the coordinates of the extremities of the zone line would necessarily be linear functions of the ratio of intercept to slope for a particular reacting pair. Such linear functions were found, confirming the hypothesis. These functions provided the coordinates for constructing theoretical zone lines for reactants of any assumed values of purity. The experimental zones fitted the theoretical zones with a goodness greater than was likely to be due to chance. It was concluded that the zone lines represented true regions of equivalence in which essentially complete precipitation of both reactants had occurred.

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THE ROLE OF MACROPHAGES IN THE ELIMINATION OF BACTERIA FROM THE MOUSE PERITONEUM

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FOR many years pathologists have believed that the mononuclear phagocytes play an important part in the resistance of the host to bacterial infection, and histological examination of mononuclear cells has shown clearly that bacteria are both engulfed and digested by these cells. For instance Robertson and Van Sant (1939) concluded that macrophages are more actively phagocytic for, and more rapidly digest, pneumococci than polymorphonuclear leucocytes particularly in lower concentrations of opsonins. These workers were also able to demonstrate phagocytosis and digestion of pneumococci by macrophages *in vitro*.

In spite of this and many other papers demonstrating intracellular digestion of bacteria by exudate macrophages, tissue culture methods were not used to study the interaction of bacteria and macrophages, until Jacoby (1944) in a short note described some potentialities of the system. Since then many workers have used such techniques notably Suter (1952, 1953) and Mackaness (1952, 1954) both of whom observed the effects of tubercle bacilli on macrophages of the rabbit and the guinea-pig. Whilst there are divergencies in the results obtained by these 2 workers they are nevertheless agreed on the major point that tubercle bacilli always multiply inside macrophages. Similar results have been reported with *Brucella* sp. by Pomales-Lebron and Stinebring (1957) and Freeman and Vana (1958). However, Rowley (1958) obtained evidence that mouse macrophages would kill an avirulent strain of *Bacterium coli* extremely rapidly in an *in vitro* system.

The most convenient source of mammalian macrophages is the peritoneal cavity. A satisfactory yield of macrophages with few polymorphonuclear leucocytes can be obtained by washing out the peritoneal cavity with a tissue culture medium without previous preparation (Rowley 1958, Freeman and Vana 1958). A logical extension of this method is to inject bacteria intraperitoneally at timed intervals before washing out the peritoneum, as conditions should then be optimal for phagocytosis. We have used this technique to study the fate of bacteria ingested by macrophages both in the peritoneal cavity and after transfer of infected cells to tissue culture flasks. In addition we have compared the bactericidal activity of macrophages from specifically immune animals and from animals with an increased level of non-specific immunity with those from normal animals.

MATERIALS AND METHODS

Strains of bacteria.—The following were used: *Bacterium coli* strains E2206, E2380 and 145 of varying virulence for mice and with different C′ sensitivities (Rowley 1954), the first

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2 strains have the antigenic structure O_{111} B_4 H_{12}, strain 145 is of unknown antigenic group, *Salmonella typhimurium* C5 a mouse virulent strain and Maaløe's avirulent strain M206 (Furness and Rowley, 1956), *Bacterium friedländeri* strain 2F (Wardlaw and Howard, 1959), which was mouse virulent, a recently isolated strain of *Pseudomonas pyocyanea* and the Oxford strain of *Staphylococcus aureus*.

All strains were kept on agar slopes in screw capped bottles at 4° from which they were subcultured as required.

Mouse strain.—All mice used were of the LAB strain of grey mice bred in this Institute. Mice were kept on the standard pellet diet 41B and weighed 18–25 g. when used.

Bacterial antisera.—Bacterial antisera were prepared in accordance with a standard method (Wadsworth 1927). When used for opsonizing bacteria they were diluted 5 times less than the agglutinating titre except where otherwise stated *i.e.* a strain agglutinating to 1/1000 was used at 1/200. Agglutination tests on sera were performed by the method of Felix and Bensted (1954). All sera were heated at 56° for 30 min. before use.

Tissue Culture methods

Flasks.—Porter flasks (Porter, Claude and Fullam, 1945) made of neutral glass and of 3·5 cm. internal diameter were used for all cultures, into each of these a " flying coverslip " was inserted. All glassware was cleaned in hot Calgon and sodium metasilicate solution followed by prolonged rinsing in distilled water as described by Hanks (1955).

Medium.—Medium No. 199 (Morgan, Morton and Parker, 1950) kindly supplied by the Biological Standards department of the M.R.C. was used. This medium contained no antibiotic and was adjusted to pH 7·2 by adding fresh 1 per cent sodium bicarbonate solution. On receipt the medium was dispensed into screw capped 20 ml. pyrex glass tubes and stored at 4°.

Peritoneal macrophages.—These were obtained in the following general manner. A mouse was killed by dislocation of the cervical spine, pinned out in a U.V. chamber and the skin reflected to expose the peritoneum. Using a 2 in. 20 gauge needle 1·5 ml. of 199 medium containing 0·5 international units of Heparin/ml. were injected into the peritoneum, and within a few seconds 1 ml. was withdrawn. This contained a total of 2–8 $\times$ 10^6 cells, and in the great majority of cases mononuclear cells accounted for 90–95 per cent of the total. This cell suspension was slowly squirted into a Porter flask containing a " flying coverslip " representing approximately 1/5 of the floor area, the flask was then flushed with a mixture of 5 per cent CO_2 and air, closed with a silicone rubber stopper and incubated at 37°. Within 20 min. the macrophages had settled on and were attached to the floor of the flask and to the coverslip and during the next 60 min. if the medium and technique had been satisfactory, were showing signs of spreading with elongated pseudopodia.

Infection of macrophages in vivo.—An overnight bacterial culture was diluted 1/10 in 199 medium and to one portion of this a quantity of heat-inactivated specific rabbit antiserum was added such that the final mixture contained approximately 5 limiting agglutinating doses. The dilution in 199 medium alone and that containing the specific antiserum were both incubated for a further 30 min. after which time they were further diluted at least 10 fold in 199 medium to give an estimated content of 10^5–10^6 organisms in 0·2 ml. This quantity of either of the two suspensions was injected intraperitoneally into each mouse of the series, which were then sacrificed after varying intervals of time and washed out with heparinized 199 medium as previously described, with the added precaution of gently mixing the peritoneal washout by partial withdrawal and reinjection several times.

The total bacterial count of each peritoneal washout was immediately determined by spreading 0·1 ml. of a suitable dilution on to a nutrient agar plate (T in Table I). One ml. from the remainder of the washout was then gently introduced into a warmed Porter flask, flushed out with 5 per cent CO_2 and air, stoppered and placed in the 37° incubator. After an interval of 15–45 min. (M) the flask was removed and the supernatant fluid pipetted out, diluted suitably and 0·1 ml. plated for a bacterial count. The " flying coverslip " with approximately 1/5 of those cells which had become attached to the glass, was hooked out aseptically, drained whilst inside the flask neck and placed upside down on a MacConkey plate (or nutrient agar in the case of *Staph. aureus*). This coverslip was then vigorously pushed around the surface of the plate with a metal spreader in order to dislodge and disrupt the macrophages. MacConkey medium was found to give the highest yield of viable bacteria for a given number of infected cells.

Mice showing non-specific immunity.—For some experiments mice were used which 48 hr. previously had been injected intravenously (i.v.) with 50 μg. of *Bact. coli* 2380 lipopolysaccharide, which treatment is known to enhance non-specific immunity. Pooled sera from a proportion of these mice failed to agglutinate a suspension of a living O_{111} B_4 strain at a dilution of 1/10.

Specifically immune mice.—Mice were immunized by 3 i.v. injections of a heat killed vaccine of *Bact. coli* 2206 at weekly intervals and used a week or more after the last injection. Blood was obtained from some of these mice and the serum from these had an average agglutinin titre of 1/100.

RESULTS

Macrophages from Normal Animals

In a typical experiment mice were injected with *Bact. coli* 2206 either diluted in 199 medium alone or diluted in 199 medium after exposure to specific antiserum. The results are collected in Table I. It is apparent that the serum treated organisms rapidly disappear from the peritoneal cavity whereas untreated organisms are hardly removed throughout the period of the experiment. In addition the disappearance of serum treated organisms continues in the Porter flasks whereas the numbers of untreated organisms are again not appreciably diminished. The difference in bacterial count between the peritoneal washouts which were used to inoculate the flasks (T) and the supernatant count (S) determined after M minutes must either be due to killing of bacteria in the supernatant or to phagocytosis. The first possibility was tested by further incubation of supernatants from which the cells had been removed, in all cases bacterial multiplication occurred. It seems probable then that the difference between the count at the time of inoculation of the flask (T) and the subsequent count on the supernatant from the flask (S) is caused by phagocytosis of bacteria. Failure to recover a number of bacteria from the cells (C) corresponding to the number phagocytosed (T–S), after incubation of the flask, must indicate killing of bacteria by the macrophages. It will be noted that, in the case where 45 min. elapsed within the peritoneal cavity in the serum treated series, the percentage of phagocytosis is smaller than in the previous flasks; we feel that this may have been due to our having allowed insufficient time for all the macrophages to become attached to the glass. The total number of organisms recovered from the cells is, of course, increased in all cases by the carry over of a small amount of the supernatant fluid on the coverslip, but we have deliberately refrained from washing the coverslips before plating in order to prevent loss of macrophages and phagocytosed bacteria by so doing. No allowance for this carry over has been made in these experiments nor does it influence the overall conclusions.

From the figures in Table I it is possible to calculate the rate of fall of the peritoneal count or half life of bacteria in the peritoneum which averages 7 min. throughout this experiment. Similarly it is possible to calculate the rate of killing (half life) of phagocytosed bacteria in the flasks, which is 9 min. in this experiment. This correlation between the 2 figures suggests that the rapid fall of the bacterial count in the peritoneum is due to killing by the phagocytes. No figures for the survival time of non-serum-treated organisms could be obtained over the period of the experiment.

The remarkable difference in the elimination rate of organisms dependent on previous serum treatment was thought to be due to the specific antibody content of the rabbit antiserum. A series of similar experiments was performed using

TABLE I.—*Phagocytosis and Survival of* Bact. coli 2206 *after Injection into the Peritoneum of Normal Mice*

Time after injection (min.)	T Total organisms recovered/ml.	Per cent inoculum recovered	Half life *in vivo* (min.)	M Time in flask (min.)	S Total organisms in supernatant/ml.	C Total organisms in cells	$\frac{100 \times C}{T-S}$ Per cent survival in cells	$\frac{100.(T-S)}{T}$ Per cent Phagocytosis	Half life *in vitro* (min.)
A—Organisms exposed to a 1/250 dilution of heated rabbit antiserum. Inoculum 380,000 organisms.									
0	276,000	108	—	40	112,000	7,500	4·5	59	9
5	265,000	104	—	40	125,000	12,500	9	53	12
10	120,000	47	9	40	6,000	2,000	1·8	95	7
15	39,000	15	5½	35	1,000	600	1·5	97	6
20	17,000	7	5½	45	1,800	1,100	7	90	12
30	18,000	7	8	35	300	2,000	11	98	11
45	4,500	1·8	7½	15	2,000	300	12	56	5
B—Organisms given without previous serum treatment. Inoculum 115,000 organisms									
0	67,000	88	—	30	103,000	850	—	—	—
5	55,000	74	—	40	57,000	700	—	0	—
10	85,000	113	—	40	81,000	1,500	—	5	—
15	67,000	88	—	40	79,000	2,250	—	—	—
20	70,000	94	—	30	70,000	2,500	—	0	—
30	64,000	85	—	40	64,000	2,500	—	0	—
45	60,000	80	—	30	50,000	2,500	25	17	15

immune mouse serum, with an agglutinating titre of 1/100, to sensitize the organisms. The object was to ascertain what was the limiting dilution of the antiserum which would still opsonize the organisms. For this purpose dilutions of mouse

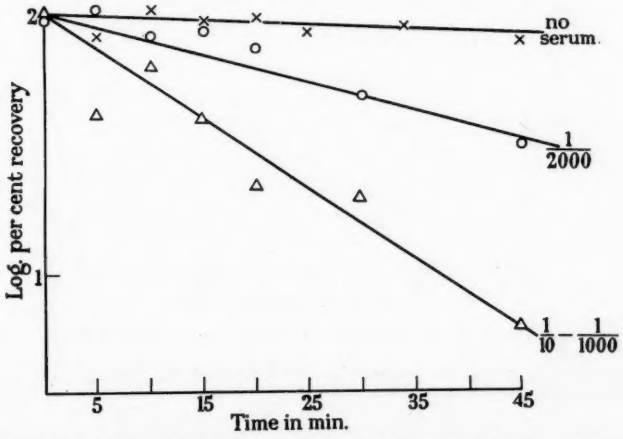


FIG. 1.—Recovery of *Bact. coli* E2206 after previous treatment with antiserum 1/10–1/2000.

serum in 199 medium were used and the dilution figures shown in Fig. 1 are those at the time of sensitization. In all cases the bacterial suspension was further diluted at least 10 fold before injection. It can be seen from Fig. 1 that diluting the antiserum to 1/250 (*i.e.* 2½ times more than its limiting agglutinating titre) does not affect the rate of removal of sensitized bacteria and even when the serum is

diluted to 1/2000 there is still some opsonic effect. The fact that normal mouse serum at a dilution of 1/10 did not change the situation from that obtained without serum treatment also supports the view that opsonization is due to specific antibody.

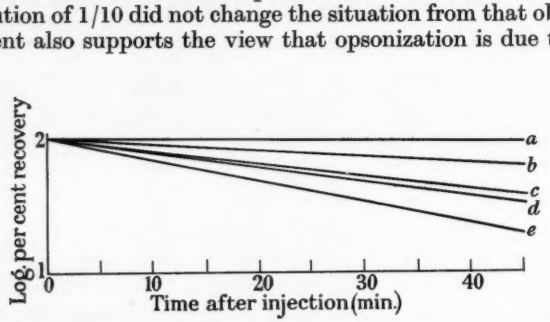


FIG. 2.—Accumulated data on the recovery of various untreated organisms from the mouse peritoneum.
(a) *Salm. typhimurium* C5, *Bact. coli* E2380; (b) *Bact. coli* 145; (c) *Staph. aureus*; (d) *Ps. pyocyanea*; (e) *Salm. typhimurium* M206.

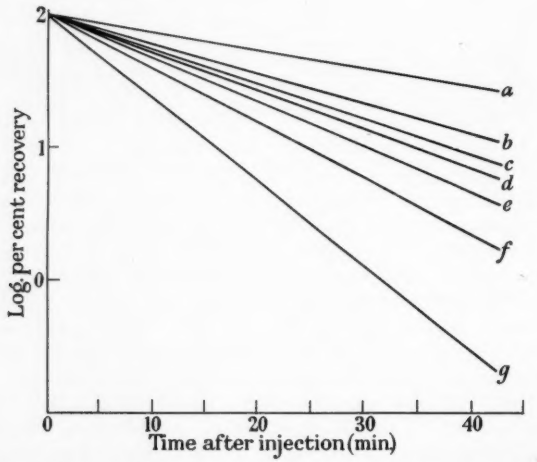


FIG. 3.—Accumulated data on the recovery of various serum treated organisms from the mouse peritoneum.
(a) *Staph. aureus*; (b) *Salm. typhimurium* C5; (c) *Bact. friedländerei*; (d) *Salm. typhimurium* M206, *Bact. coli* 145; (e) *Bact. coli* E2206; (f) *Bact. coli* E2380; (g) *Ps. pyocyanea*.

In Figs. 2–3 the percentage recoveries of various organisms after injection into the mouse peritoneum are plotted against time. There are considerable variations with different organisms and some are removed rapidly without previous serum treatment. Although the removal of *Ps. pyocyanea* is very greatly increased by serum treatment the rates for *Salm. typhimurium* M206 and *Staph. aureus* show little change. In order to see if there was any correlation between virulence and rate of removal the pair of *Salm. typhimurium* organisms, which differ greatly in mouse virulence, were investigated in more detail. Figs. 4–5 show that these

organisms, once they have been serum sensitized. are removed in an indistinguishable manner. On the other hand without serum treatment the number of virulent organisms recovered remains practically constant whereas the numbers of the avirulent organisms have decreased by more than 99 per cent within 2 hr.

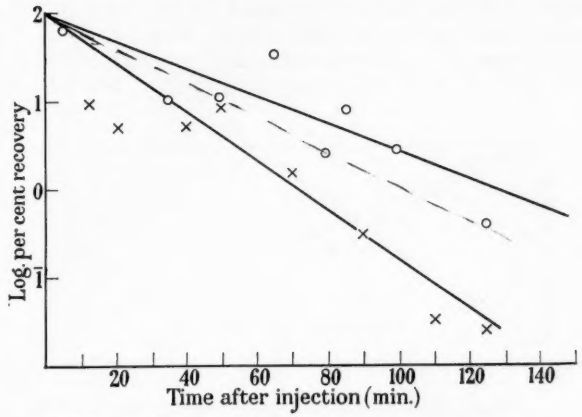


FIG. 4.—Recovery of *Salm. typhimurium* M206 from the mouse peritoneum.
×——× Organisms previously treated with antiserum.
○——○ Untreated organisms.

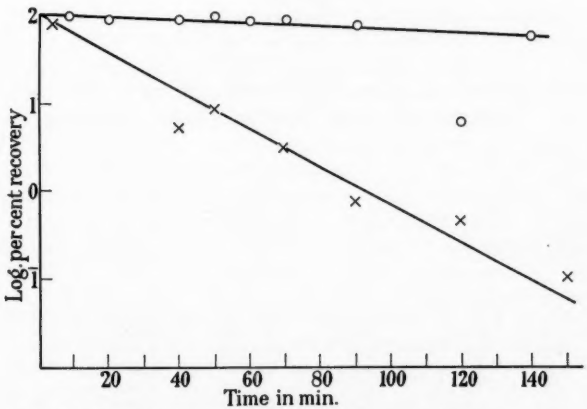


FIG. 5.—Recovery of *Salm. typhimurium* C5 from the mouse peritoneum.
×——× Organisms previously treated with antiserum.
○——○ Untreated organisms.

Macrophages from Specifically Immune Mice and Mice with an Enhanced Degree of Non-specific Immunity

Mice immunized by 3 i.v. injections at weekly intervals with a killed suspension of *Bact. coli* O_{111} B_4 H_{12}, were used to repeat the type of experiment shown in

Table I, using *Bact. coli* 2380. The results are shown in Fig. 6 where the elimination of non-serum-treated *Bact. coli* 2380 from the normal mouse peritoneum is included for comparison. It is clear that both the serum treated and untreated organisms are removed at a similar rapid rate from immune mice and reference to Table II shows that this can be accounted for by the rapid phagocytosis of non-serum-treated organisms by immune macrophages.

An enhanced degree of non-specific immunity was induced in mice by an i.v. injection of 100 μg of a *Bact. coli* lipopolysaccharide. After 48 hr. the rate of

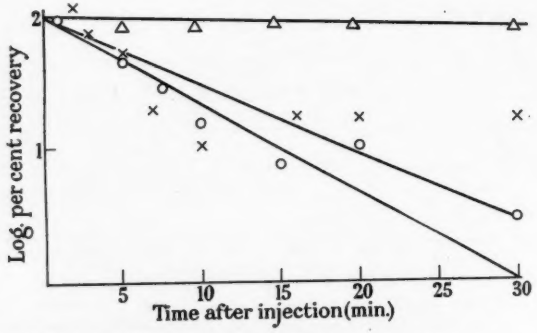


FIG. 6.—Recovery of *Bact. coli* E2380 from specifically immune mice.
×——× Organisms previously treated with antiserum.
○——○ Untreated organisms.
△——△ Untreated organisms in normal mice.

TABLE II.—*Phagocytosis and Survival of* Bact. coli 2380 *after Injection into the Peritoneum of Specifically Immune Mice*

Time after injection (min.)	T Total organisms recovered/ml.	Per cent inoculum recovered	Half life *in vivo* (min.)	S Total organisms in supernatant from flasks/ml.	C Total organisms in cells	$\frac{100 \times C}{T-S}$ Per cent survival in cells	$\frac{100\ (T-S)}{T}$ Per cent phagocytosis
A—Organisms given without previous serum treatment. Inoculum $1 \cdot 2 \times 10^6$ organisms							
2	692,000	87	10	420,000	5,000	1·9	39
5	260,000	32·5	2·5	55,000	10,000	4·9	79
7·5	167,000	21	3·5	100,000	2,000	3·0	40
10	93,000	11·5	3·5	16,000	2,000	2·6	83
15	45,000	5·5	4	4,000	1,250	3·1	91
20	60,000	7·5	5·5	3,000	1,250	2·2	95
30	18,000	2·2	5·5	230	370	2·1	98
B—Organisms previously exposed to dilute specific antiserum. Inoculum 600,000 organisms							
2	350,000	88	9	140,000	25,000	12	60
3	230,000	57·5	4	23,000	50,000	24	90
5	150,000	37·5	3·5	40,000	25,000	23	73·5
7	55,000	14	2·5	25,000	10,000	33	62
10	31,000	8	2·5	9,000	2,250	10	71
15	58,000	14·5	6	13,000	5,000	11	78
20	50,000	12·5	6·5	5,360	2,500	5·5	89
30	50,000	12·5	10	3,700	500	1·1	92

removal of *Salm. typhimurium* C.5 was determined in comparison to the rate found in normal mice. If the results in Fig. 7 are compared with Fig. 5 the rates of removal of serum-treated organisms are indistinguishable. However, untreated organisms are removed more rapidly by non-specifically immune mice.

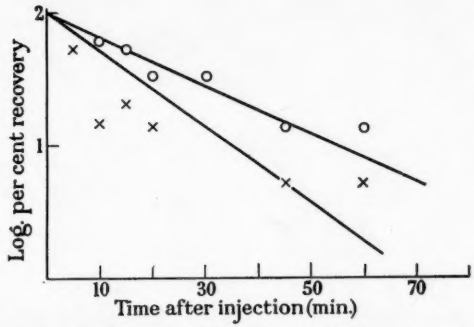


FIG. 7.—Recovery of *Salm. typhimurium* C5 from mice injected 48 hr. previously with 50 μg. *Bact. coli* E2380 lipopolysaccharide.
×——× Organisms previously treated with antiserum.
○——○ Untreated organisms.

The Injection of Serum Treated Organisms into Normal Mice

Because opsonization with specific antiserum led to rapid removal of *Salm. typhimurium* C.5 from the mouse peritoneum, it seemed that this procedure might alter the ability of this organism to produce disease in the host. Table III shows the mean time to death of mice following intraperitoneal injection of various doses of serum-sensitized and untreated *Salm. typhimurium* C.5. The mean time to death was in all cases longer after the administration of serum sensitized organisms. Fig. 8 represents the survivors of 2 groups of 30 mice at varying intervals after injection of 50 *Salm. typhimurium* C.5. The time to death following administration of serum treated organisms was prolonged and 11/30 (37 per cent) of mice survived as compared to 0/30 with untreated organisms. Thus it is clear

TABLE III.—*The Effect of Exposing* Salm. typhimurium *C5 to Dilute Antiserum on the Subsequent Lethality of the Organisms for Mice*

Challenge dose	Mean time to death (days)	
	Serum treated organisms	Untreated organisms
10^6	6·8	3·4
10^5	8	4·6
10^4	9·4	7·7
10^3	11·8	5·4
10^2	18·4	5
10	24·4	15

Each group consisted of 10 mice.
Maximum survival time was counted as 28 days.

that opsonized organisms have less ability to cause disease in the mouse than normal organisms, although antibody itself has no effect on the viability of bacteria or on their ability to multiply.

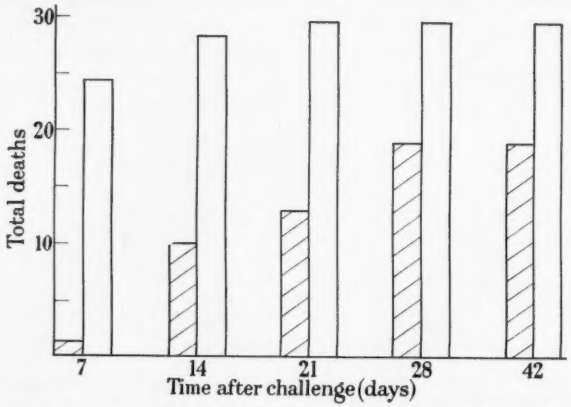


FIG. 8.—Histogram of total cumulative deaths from *Salm. typhimurium* C5 infection in mice. Thirty mice challenged in each group with 50 organisms I.P.

□ Untreated organisms.
▨ Organisms previously treated with antiserum.

DISCUSSION

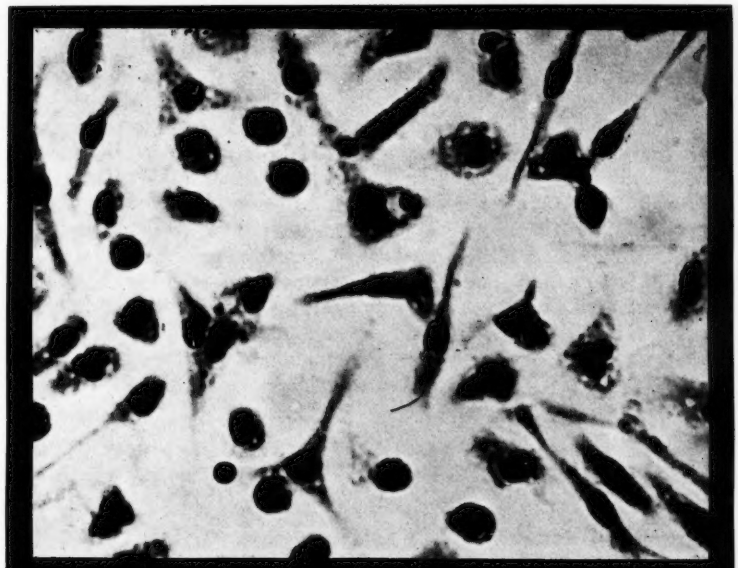
From the data presented here several conclusions can be drawn. The finding that most gram-negative bacteria without serum treatment are not removed appreciably from the peritoneum within $2\frac{1}{2}$ hr. (Fig. 2) is in agreement with the early work of Buxton (1906). In his experiments a saline suspension of approximately 10^9 typhoid bacteria injected intraperitoneally into rabbits was found to be so slowly removed that the average recovery of bacteria from the peritoneum after 6 hr. was 10^8 and even 24 hr. later 10^6 bacteria could still be recovered. Whether these bacteria were free or inside phagocytes was not determined. On the other hand the extremely rapid removal of most serum-treated bacteria (Fig. 3) indicates that lack of removal in the absence of serum treatment is due to the opsonin content of the normal peritoneal fluid being a limiting factor. Opsonization of the bacteria by antiserum is followed by rapid killing and intracellular digestion of the organisms.

The question arises whether this bactericidal effect might not be due to the 5–10 per cent polymorphonuclear cells which are present in the peritoneal lavage. We feel that the macrophages are almost certainly responsible for the effects reported here, since the cell populations which become attached to the glass in the Porter flasks are almost entirely macrophages Fig. 9 and nevertheless exhibit

EXPLANATION OF PLATE.

FIG. 9.—Field from a mouse macrophage culture. ×800 (Hanset's stain).

FIG. 10.—Mouse macrophages with intracellular bacteria undergoing digestion. ×1500 (Hanset's stain).



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very similar bactericidal powers to the mixed cell population of the peritoneal cavity. In addition by heavily infecting cell suspensions, bacteria may be observed inside mononuclear cells and the process of digestion may be followed. Fig. 10.

In our experiments probably the most conclusive evidence that the removal of organisms from the peritoneum is due to intracellular killing, is the consistent finding that this bactericidal process continues in the Porter flasks at rates which are very similar to the *in vivo* rates and Table IV summarizes our findings in this respect. It has been our experience that the very minimum of handling of the macrophages is necessary if their bactericidal powers are to be preserved in full. Changing the medium or washing by centrifugation both decreases the rates at which the macrophages kill bacteria (Rowley and Whitby, unpublished). It is possible that previous workers following the interactions of bacteria and macrophages may have failed to demonstrate bacterial killing for this reason, since in many of the studies the macrophages were centrifuged and washed with several changes of cold medium before being infected.

TABLE IV.—*Accumulated Data on Phagocytosis and Rate of Removal of Organisms from the Mouse Peritoneum and Survival Time of Phagocytosed Organisms* In Vitro *in Porter Flasks*

	Serum treated				Non-serum treated			
Organism	Number of experiments	Half life (min.) *In vivo*	Half life (min.) *In vitro*	Per cent phagocytosis	Number of experiments	Half life (min.) *In vivo*	Half life (min.) *In vitro*	Per cent phagocytosis
Ps. pyocyanea	2	5	6–12	86	2	26	5–15	44
Bact. coli 2206	8	9	5–12	88	2	60	—	4
Bact. coli 2380	2	7·5	4–10	94	2	60	—	18
Bact. coli V5	1	10	4½–9	81	1	45	—	2
Salm. typhimurium M206	2	10	6–12	88	2	18	6–15	24
Salm. typhimurium C5	3	12·5	7–15	94	3	60	—	9
Bact. friedländeri	2	11·5	5–15	86	1	60	—	2
Staph. aureus	1	22·5	12–16	94	2	27·5	13–17	80

A further feature of this work which we feel needs emphasis is the absence of antibiotics in any of our experiments. Notwithstanding the evidence that streptomycin does not give as high an intracellular concentration as exists in the extracellular fluid, there will always be some suspicion that antibiotics in the tissue culture medium might exert some effect on susceptible intracellular bacteria.

Bactericidal Effect against different Bacterial Strains

Although all the strains tested can be shown to be susceptible to the bactericidal action of macrophages, there are considerable variations in the speed with which this occurs. The 50 per cent survival time *in vivo* varies from 5 min. in the case of *Ps. pyocyanea* to 23 min. with *Staph. aureus*. These half-lives are of the same order as the 7–8 min. given by Wilson, Wiley and Bruno (1957) for the half-life of Group A Streptococci after phagocytosis by human or mouse neutrophils.

The relative speeds of removal of different strains with the exception of *Staph. aureus* may show some correlation with their mouse virulence. Thus the virulent

strain of *Salm. typhimurium*, the virulent *Bact. friedländeri* and the virulent *Bact. coli* strains are all removed at less than half the rate of *Ps. pyocyanea* Fig. 3. It is also clear that some avirulent strains can be rapidly destroyed without previous serum treatment.

With these strains i.e. *Ps. pyocyanea*, M206 and *Staph. aureus*, the half-lives of the bacteria whilst in the peritoneum were considerably longer than those obtained following serum treatment. Since, however, the amounts of phagocytosis in the absence of serum treatment were always much lower, it is probable that the rates of phagocytosis were limiting the overall bactericidal effect. This likelihood is strengthened by calculating the half-lives of the organisms inside the macrophages after plating in the Porter flasks. It can be seen (Table IV) that the rates of killing of the 3 strains in question were approximately the same *in vitro* irrespective of serum treatment.

The results with *Staph. aureus* Oxford strain deserve further mention since they are unique and differentiated from all the gram-negative bacteria by the fact that serum-sensitization made no significant difference to the rates of removal. This is in direct contrast to the results of Nanni (1958) who used guinea-pig peritoneal leucocytes to study opsonic factors necessary for the uptake of *Staph. aureus* Oxford strain ; he found a four-fold increase in the numbers of intracellular bacteria engulfed in the presence of guinea-pig serum and strongly implicated properdin as an opsonin. On the other hand our colleagues Wardlaw and Howard (1959), using a rat liver perfusion technique found that *Staph. aureus* Oxford strain was rapidly removed by the Kuppfer cells during perfusion but that there was rather less uptake in the presence of rat serum than in its absence.

Results with Specifically Immune Mice and with those Treated with Bacterial Lipopolysaccharides

Specifically immune animals, as might be expected can rapidly phagocytose non-sensitized intraperitoneal organisms, presumably because the peritoneal fluids contain sufficient opsonizing antibody for the purpose. The *in vivo* half-life (Table II) of non-sensitized organisms in specifically immune mice is 4·0 min. compared to 7·5 for the removal of sensitized organisms by normal mice. Whether this increased efficiency is a function of the specifically immune macrophages themselves, at least in part, is not clear from the present results and analysis of this may be easier in *in vitro* studies.

The antibacterial powers of the macrophages against all the bacteria examined are so striking and rapid that one wonders how organisms are ever able to overcome them. Insufficient concentration of opsonic factors at the site of infection clearly provides a situation where for a time at least the organisms can resist these host defences. It is possible that some hosts exist where macrophages are deficient in ability to destroy ingested organisms. Some slight support for this may be found from our results, in which the data from an occasional mouse is seen to diverge from the mean curve much more than the rest. Where such a point indicates greater bactericidal effect than the mean this probably indicates that the animal possessed some specific immunity (Fig. 5), on the other hand defective killing in the presence of adequate phagocytosis, which is occasionally found, could be due to defective intracellular antibacterial systems.

Mice given bacterial lipopolysaccharide 48 hr. previously behaved, so far as

their ability to remove and destroy intraperitoneal organisms, in a similar manner to the specifically immune mice mentioned above. One important difference was that no specific agglutinating antibody was detectable in the serum of the lipopolysaccharide-treated mice against strain C5, whereas that from the specifically immunized mice agglutinated the homologous strain *Bact. coli* 2380 at a dilution of 1/100. For this reason it seems unlikely that the increased phagocytic and bactericidal activity of the macrophages from non-specifically immune mice, is in any way related to serum antibody content.

In vivo *considerations*

The elimination studies of Buxton (1906) indicate that a small proportion of the intraperitoneal organisms are constantly leaking into the circulation from which they reach the liver, spleen etc ; the more organisms there are in the peritoneum the more will reach these organs. More recently Schneider and Zinder (1956) have demonstrated with *Salm. typhimurium* infections that there is a relationship between the numbers of bacteria in the spleen at 48 hr. and the probability of death after several days. It seems likely that the present results can account for the difference in virulence between the strains C5 and M206 solely on the basis of the numbers of organisms of the non-sensitized strains which survive several hours in the peritoneum.

Since antiserum treatment, particularly of the virulent strains like C5, enormously enhances the initial destruction of the bacteria when injected into mice, one would expect on the basis of Schneider and Zinder's work that the same serum treatment would in effect reduce the apparent virulence of an organism for mice. Table III shows that this expectation is well founded, the mean time to death from serum-sensitized organisms being much longer at all dose levels than that from normal unsensitized organisms. Moreover, reference to the histogram in Fig. 8 shows that besides delaying the mean time to death, serum treatment of the organisms resulted in a large percentage of the animals surviving the infection completely. We attribute this protective effect to the rapid bactericidal activity of the peritoneal macrophages which under these conditions are able to ensure that very few, if any, viable organisms escape from the peritoneum.

SUMMARY

A simple method is described, by which macrophages from the mouse peritoneum may be used to study their interactions with various bacteria. When organisms have been opsonized with specific antibody before injection into the mouse, they are seen to be rapidly ingested and destroyed *in vivo* and *in vitro*. Untreated bacteria are not removed to any appreciable extent unless phagocytosed, and are destroyed by macrophages in proportion to the amount of phagocytosis that has occurred.

Specifically immune mice eliminate untreated organisms more rapidly than do normal mice and this is again proportional to the amount of phagocytosis.

A virulent strain of *Salm. typhi-murium* when sensitized by antiserum before injection was found to be less lethal for mice.

We gratefully acknowledge a grant from the Medical Research Council towards the expenses of this work. We wish to thank Dr. D. G. Evans and Dr.

D. Magrath of the M.R.C. Biological Standards Division for generously supplying the 199 Medium. During part of this work one of us (J.L.W.) was supported by a grant kindly given by Merck & Co. Inc.

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DUODENITIS AND DUODENAL ULCERS PRODUCED IN RATS BY PANTOTHENIC ACID DEFICIENCY

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In previous papers mucosal lesions, each characteristic of one of the 3 subdivisions of the stomach were described in rats kept on various kinds of nutritional deficiencies (Berg, 1942 ; Zucker, Berg and Zucker 1945*a* and *b*). In none of these experiments did the duodenum show abnormalities. Studies with different members of the vitamin B-complex reveal that pantothenic acid deficiency produces duodenitis and duodenal ulcers. The nature and incidence of these lesions are described in the following report.

METHODS

Rats of both sexes of the Mendel-Sherman strain were used. The animals were in 2 age groups : 43 older ones, 37 females, and 6 males, with an initial age averaging 355 (229–620) days, and 23 younger rats, all males, with an average age of 101 (54–119) days. For the older animals the average experimental period was 101 (67–123) days and in the younger age group the average experimental time was 110 (84–135) days.

All the known nutritional factors necessary for growth except pantothenic acid were included in the diet, which had the following composition per 100 g. : vitamin " free " casein (Labco) 18·0 ; cerelose 71·9 ; salt mixture 6·1 (Ca 1·0, P 0·55) ; cottonseed oil 2·0 ; celluflour 2·0. Incorporated in the oil were 10 mg. α-tocopherol and 1500 units of vitamin A, as carotene. The celluflour contained : thiamin HCl and pyrodoxine HCl 1 mg. each ; riboflavin 2 mg.; niacin 4 mg. and 2-methyl-naphthoquinone 0·5 mg.

The rats were killed with ether and complete autopsies were performed immediately after death. The stomach and upper small intestine were opened and stretched out with pins on cardboard. After inspecting the mucosa in the fresh state, the specimen was immersed in a 4 per cent solution of formaldehyde and examined again 24 hr. after fixation. Tissues were imbedded in paraffin and prepared for microscopic study by staining with haematoxylin and eosin. Data on incidence and time of onset of duodenal lesions were based on microscopic findings.

RESULTS

General effects of pantothenic acid deficiency

After variable periods, characteristic signs of the deficiency such as spectacle alopecia, pigment on the nose and hairs of the snout and around the eyes, and haemorrhagic necrosis of the adrenals appeared. Previously undescribed findings are duodenitis and duodenal ulcers. These lesions were never seen in any of hundreds of normal controls, nor in rats kept on diets deficient in the other compo-

nents of the vitamin B-complex. The remainder of the small intestine and the large intestine were uninvolved.

Gross duodenal changes in pantothenic acid deficiency

The gross appearance of the duodenum varied with the type of lesion. In the presence of acute duodenitis the wall was swollen and oedematous. The mucosa was congested and in some places had a grey, seared appearance. Elsewhere there were white necrotic patches surrounded by smooth depressions and granular areas studded with tiny haemorrhagic and white spots. Usually multiple small superficial haemorrhagic ulcers were also present (Fig. 1).

In the subacute stage the mucosa, which normally has a velvety appearance, showed smooth translucent depressions beginning at the pyloric region and extending distally as shallow grooves (Fig. 2). In some instances replacement of mucosal pattern by a flat smooth surface was extensive and only scattered islands of intact lining remained.

At the site of an ulcer the duodenal wall was indurated and the serosa was congested. Defects caused by perforation were sealed by fibrous tissue and adherent pancreas (Fig. 3). Fibrinopurulent peritonitis due to leakage from incompletely sealed perforations occurred twice. Bleeding was common and in 2 instances death was due to haemorrhage from an eroded vessel in the base of an ulcer.

Ulcers were frequently multiple. Acute lesions varied in size from small, superficial defects (Fig. 1) to deeper ones measuring up to 1·0 × 0·4 cm. Chronic ulcers had firm margins and measured from 0·3 × 0·5 cm. to 2·0 × 1·5 cm. (Fig. 3).

Microcopic appearance of duodenal lesions

Duodenitis.—The earliest discernible change was a break in continuity of epithelium covering the tip of a villus. An exudate containing polymorphonuclear leucocytes filled the defect and the lesion resembled the " leistenspitzen erosionen " described by Konjetzny (1928). More advanced changes consisted of necrosis of villi with acute inflammation of the supporting stroma and in extreme cases only remnants of villi covered by a fibrinopurulent exudate remained (Fig. 4). After subsidence of the acute stage, repair sets in and mucosal defects are covered by a flat layer of cuboidal epithelium (Fig. 5).

Duodenal ulcers.—As a rule acute ulcers occurred in areas of mucosal necrosis while chronic ulcers were usually found in areas of mucosal repair (Fig. 6). Small acute defects were limited to the mucosa and muscularis mucosae. Larger acute ulcers extended to the deeper layers and were accompanied by an acute inflammatory reaction. Chronic ulcers penetrated the duodenal wall and the defects were bridged by dense fibrous tissue and adherent pancreas (Fig. 6).

Incidence of duodenal lesions

With the exception of chronic ulcers, the incidence of duodenal lesions was about the same in young rats and in older ones. Various stages of duodenitis were seen in 90 per cent of animals. Acute duodenitis including superficial ulcers occurred in 50 per cent. Mucosal defects covered by newly regenerated epithelium were observed in 40 per cent. The overall incidence of all types of ulcers was 60 per cent. Acute penetrating ulcers were found in 35 per cent of rats, while chronic

ulcers developed in 13 per cent of young animals and in 26 per cent of older ones. Antral and fundal lesions of the kind described previously in nutritional gastritis (Berg, 1942) were found in 10 per cent.

Time of onset of duodenal lesions

A separate experiment was performed to determine the latent period before the appearance of duodenal lesions. Twenty-three male rats with an initial age of 17 weeks were given the pantothenic acid deficient diet and were divided into small groups which were examined after 4, 8, 12 and 15 weeks. No changes in the duodenum were seen until the twelfth week. At this time, out of 8 animals, 4 showed signs of epithelial repair and in one of these rats there were also acute and chronic ulcers. After 15 weeks ulcers were found in 3 out of 4 rats. In 2 instances the ulcers were of the acute penetrating type and in the third the ulcer was chronic.

Adrenal lesions and duodenal ulcers

In the time experiment just cited one rat with haemorrhagic necrosis of the adrenals had duodenitis while 4 others with relatively normal glands had penetrating ulcers. In the larger series, swollen, haemorrhagic adrenals were found in 8 animals with ulcers, but the same degree of adrenal damage was observed in 6 rats with no ulcers.

DISCUSSION

These experiments show that pantothenic acid is required by the rat to maintain an intact duodenal mucosa. The high incidence of duodenitis and the constant finding of ulcers in degenerated epithelium strongly supports the hypothesis that mucosal changes are precursors to ulcers.

Though the experimental ulcers are similar to human ones, there is little likelihood of a spontaneous pantothenic acid deficiency in the human diet because of the wide distribution of this factor in natural foods. Nevertheless, mechanisms interfering with pantothenic acid metabolism may induce a deficiency despite an adequate supply in the diet (Wright and Welch, 1943).

Pantothenic acid deficiency affects chiefly the duodenal mucosa and the adrenal cortex. Lesions in both are similar in both in so far as the changes are degenerative, but there is no correlation between the 2 as regards severity.

SUMMARY

Pantothenic acid deficiency in rats produces duodenitis and duodenal ulcers. Lesions develop with about equal frequency at different ages in both sexes. The latent period before the appearance of lesions is between 8 and 12 weeks of the deficiency. There is no parallelism between duodenal ulcers and adrenal degeneration.

Acknowledgement is made to Drs. T. F. Zucker and L. M. Zucker for their cooperation in this work. This investigation was supported by a research grant from the National Institutes of Health, U.S. Public Health Service.

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EXPLANATION OF PLATES

FIG. 1.—Acute diffuse duodenitis. Mucosa swollen, and necrotic patches in smooth depressed areas. Several small acute ulcers in post-pyloric region.

FIG. 2.—Smooth mucosal depression in post-pyloric region continued distally as a shallow groove. Ulcer in centre of flat area. Healing stage of duodenitis.

FIG. 3.—Large chronic perforated duodenal ulcer with dense fibrous tissue and adherent pancreas in base.

FIG. 4.—Acute diffuse duodenitis. Inflammation and necrosis of mucosa beginning in pyloric region at transition from normal antral mucosa where there is a superficial ulcer. × 18.

FIG. 5.—Abrupt change from intact antral mucosa to defect in duodenal mucosa covered by a flat layer of regenerating epithelium. Brunner's glands are dilated and irregular. This photomicrograph is a higher magnification of the blocked-in section in Fig. 6. × 66.

FIG. 6.—Mucosal defect over Brunner's glands covered by regenerating epithelium which extends to edge of chronic perforated ulcer with dense fibrous tissue and adherent pancreas forming base. × 6.

All histological sections were stained with haematoxylin and eosin.

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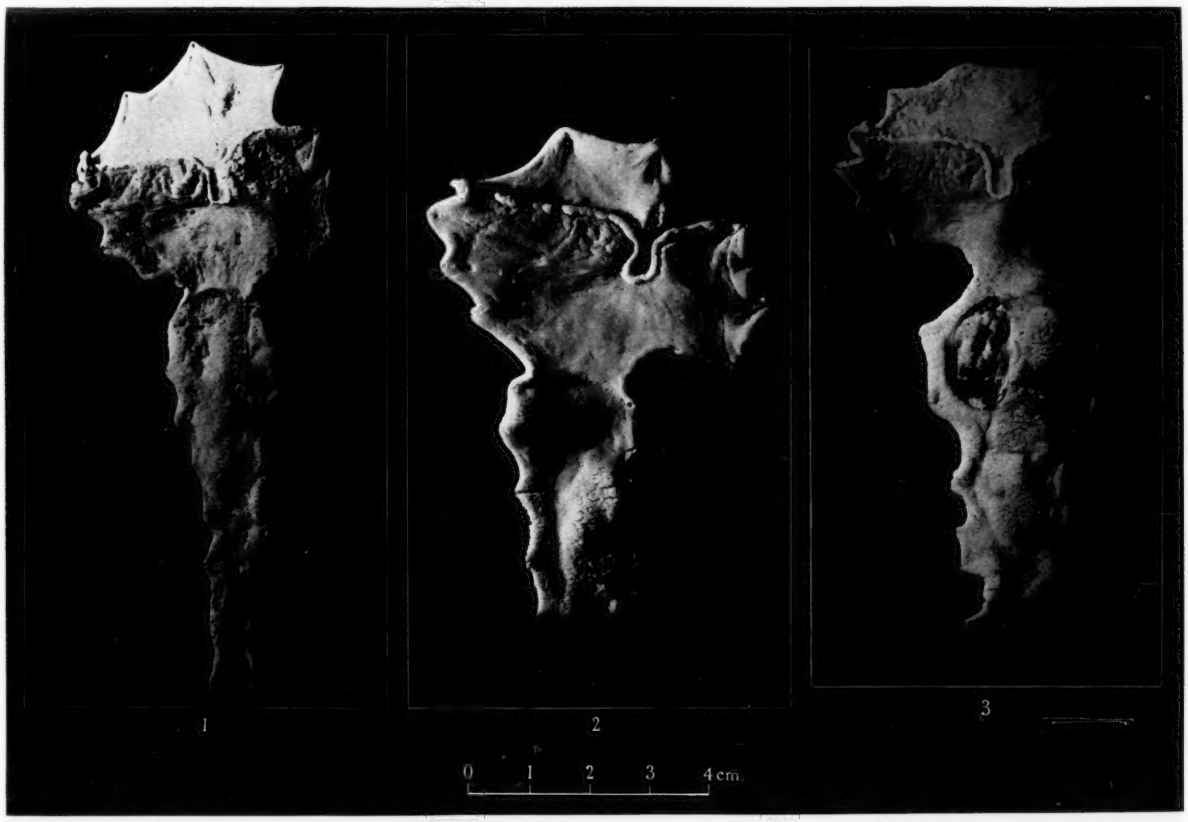

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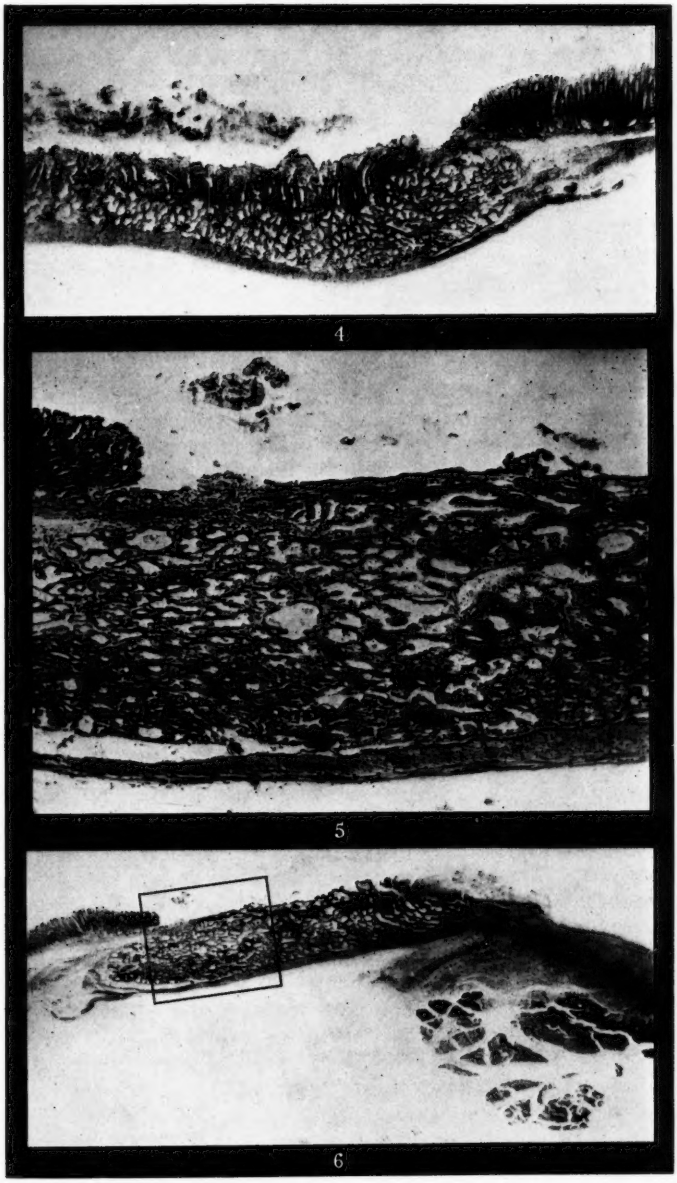

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MYOSIN AND ACTIN FROM MYOFIBRILS OF VITAMIN E DEFICIENT RABBITS

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It has been shown that the content of myosin and actomyosin in muscle extracts from vitamin E deficient rabbits is decreased, compared to normal controls (Aloisi, 1951 ; Bonetti, Aloisi and Merucci, 1952 ; Crepax, 1952 ; Azzone and Aloisi, 1958). The amount of myosin which can be extracted under these conditions is more markedly decreased than is the actomyosin.

When the usual methods are used for the extraction of whole muscle, it is difficult to relate the values of the extracted proteins to the true " muscle phase "; the methods of estimating the relative volume of the ' interstitial phase " and the amount of necrosis in muscle fibres do not seem to be completely reliable. It is also difficult to solve the problem of whether less myosin is present in the muscle fibres or whether the extractability or solubility is changed, so that a smaller amount is extracted or is separated from the extracts by the usual potassium chloride fractionation.

Extraction of myofibrils by Perry's method appeared to facilitate the investigation considerably. It is possible that a process of separation which was devised for normal myofibrils introduces some degree of selection with the result that the most altered myofibrils are discarded during the differential centrifugation ; this would only increase the significance of any change to be found in the final preparations.

In an earlier communication (Corsi, 1957*a*) it was shown that the yield of myofibrils from dystrophic rabbit muscle is reduced as compared with preparations obtained from normal rabbits ; reduction was proportional to the relative increase of connective tissue and to the extent of necrosis as far as could be estimated by histological examination. A normal amount of protein could be extracted from myofibrils by high ionic strength media, but the amount of myosin that could be purified from the KCl-pyrophosphate-$MgCl_2$ extracts was much reduced.

The electrophoresis diagrams of the extracts pointed to the possibility that a larger proportion of myosin might be bound with actin than was the case with myofibrils from normal muscle. The present paper is concerned with a further investigation of myosin and actin in dystrophic muscle.

MATERIALS AND METHODS

Young rabbits were fed on the diet of Houchin and Mattill (1942) slightly modified by Aloisi, Ascenzi and Bonetti (1952*a*) and killed when they were no longer able to walk. The control rabbits were fed on the same diet with α-tocopherol added (20 mg. twice weekly).

Another group of animals was fed on wheat bran and vegetables. The number of animals in each group was 20, 4 and 11 respectively.

The method for the preparation of the myofibrils was that used in previous studies (Perry and Corsi, 1958). Immediately after the end of the preparation, the myofibrils were extracted for 2½ hr. with a high ionic strength medium. Two kinds of media were used: 0·5 M KCl 0·04 M K phosphate buffer (pH 7·5) or 0·47 M KCl 0·1 M phosphate buffer (pH 6·2) and 0·01 M $Na_2P_2O_7$, 0·001 M $MgCl_2$. The extract was separated by centrifugation (20 min. at 29,000 g) and dialysed against 0·0325 M KCl containing 0·005 M K phosphate buffer (pH 7·1) in order to separate the "extra-protein" fraction (Szent-Györgyi, Mazia and Szent-Györgyi, 1955; Corsi, 1957*b*). The precipitate was centrifuged (for 5 min. at 25,000 g) and redissolved by increasing the ionic strength up to 0·5 with KCl.

Fractionation at 0·3 μ was carried out by diluting the solution with distilled water; pH was always about 6·5–6·7. The precipitate was separated by centrifugation after 18–20 hr. at 2°–3° and the absorption of the supernatant was measured with the Beckman spectrophotometer at 278 mμ.

The salting-out experiments with $(NH_4)_2SO_4$ were carried out by a method similar to that described by Tenow and Snellman (1954): the protein solution was dialysed against 0·6 M KCl containing 0·05 M K phosphate buffer (pH 6·9) and mixed with saturated $(NH_4)_2SO_4$ neutralized with NH_3; after 18–20 hr. at 2°–3° the supernatant was separated by centrifugation and absorption was measured at 278 mμ.

Electrophoresis was carried out with the Perkin-Elmer apparatus (model 38A), the solution was dialysed against 0·26 M KCl containing 0·05 M K phosphate buffer (pH 6·9).

Viscosity measurements were carried out at 0° with the Ostwald viscometer.

Nitrogen was estimated by the micro-Kjeldahl technique.

The bound nucleotides were isolated by a method similar to that described by Perry (1952). One volume of cold 60 per cent trichloroacetic acid (TCA) was mixed with 5 volumes of the myofibril suspension. After standing 10 min. at 0° the myofibrillar precipitate was separated by filtration and the acid-labile phosphate (the inorganic phosphate liberated upon heating with N HCl for 7 min. at 100°) was estimated in the clear filtrate by the method of Fiske and SubbaRow (1925). Samples of the TCA filtrate were extracted three times with ether to remove the TCA and finally freeze-dried. The dry residue was dissolved with as little distilled water as possible; ATP and ADP were separated by paper chromatography as described by Krebs and Hems (1953): if only 0·005 ml. of solution were put on the paper, salts did not appreciably interfere with the separation. For a more accurate determination of ATP, samples of the nucleotide preparations were incubated with myosin (freed from myokinase by 3 precipitations) in the presence of 0·05 M TRIS buffer (pH 7·4) and 0·005 M $CaCl_2$ (final volume 3 ml.). Incubations were carried out for 30 min. at 33°.

RESULTS

When myofibrils were extracted with 0·5 M KCl (pH 7·5) without pyrophosphate and $MgCl_2$, 30–40 per cent of the myofibrillar protein passed into solution; the composition of the extracts freed from the "extra-protein" was somewhat variable and no appreciable difference could be detected in the vitamin E deficient animals in comparison with the normal ones: actomyosin appeared to be the main component in all cases.

On the contrary, when the myofibrils were treated with KCl-pyrophosphate-$MgCl_2$, a much larger amount of protein passed into solution and a significant difference could always be detected in the composition of the extracts from normal and dystrophic rabbits.

In the solutions prepared from normal rabbits (fed on the diet of Houchin and Mattill (1942) supplemented with α-tocopherol, or on natural complete foods), electrophoresis showed that myosin was the main component; as a rule an actomyosin peak could not be detected (Fig. 1). The amount of protein precipitated at 0·3 μ was usually less than 20 per cent of the total. The viscosity number

$Z\eta$ was 0·20–0·22 and the ATP sensitivity ranged between 10 and 20. In most cases the salting out experiments with $(NH_4)_2SO_4$ did not show exactly the precipitation range of actomyosin because between 20 and 30 per cent saturation a modest increase of the optical density of the solutions usually occurred. Although the protein material precipitated at 35 per cent saturation (where a marked break indicates the end of the precipitation zone of actomyosin) was not represented only by actomyosin, the values obtained were thought to be useful for comparison ; they were always less than 40 per cent of the total protein content of the solutions.

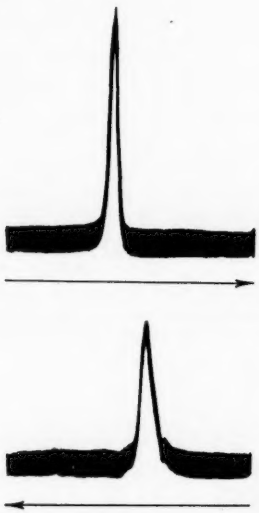

FIG. 1.

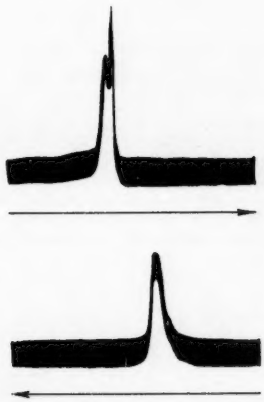

FIG. 2.

FIG. 1.—Electrophoresis diagram of a fraction extracted from isolated myofibrils of a normal rabbit with KCl-pyrophosphate-$MgCl_2$. The extract was then freed of the extra-protein and finally dialysed against 0·26 M KCl containing 0·05 M K phosphate, pH 6·9. After 720 min. The upper is the ascending boundary.

FIG. 2.—Electrophoresis diagram of a fraction extracted from isolated myofibrils of a dystrophic rabbit. The extract was obtained and treated by the method described for Fig. 1. After 480 min. The upper is the ascending boundary.

In the solutions prepared from dystrophic rabbits electrophoresis always showed a well marked peak which moved faster than myosin (Fig. 2). The amount of protein precipitated at 0·3 μ was always more than 35 per cent of the total. $Z\eta$ values were close to 0·30 and the ATP sensitivity was comparatively high (up to 41). The protein material precipitated by 35 per cent saturated $(NH_4)_2SO_4$ was always more than 40 per cent of the total. In one case (experiment no. 60D) it was possible to recognise the precipitation range of actomyosin and the value for actomyosin calculated from the salting-out curve compared well with the amount of protein precipitated at 0·3 μ : 47·7 per cent and 46·6 per cent respectively (Figs. 3 and 4).

Electrophoresis showed that neither the material precipitated between 35 per cent and 43 per cent saturation of ammonium sulphate nor the material isolated by 0·3 M KCl fractionation were pure proteins, but both appeared to be made up mainly of actomyosin ; two peaks could be seen, the mobility of which

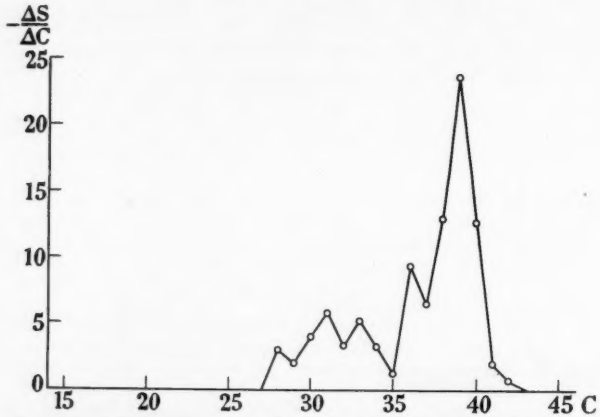

FIG. 3.—The derived solubility curve of a fraction extracted from isolated myofibrils of a normal rabbit with KCl-pyrophosphate-$MgCl_2$. The extract was then treated as described in the text. S is extinction of the solution at 278 mμ (extinction at the start being 100). C is per cent saturation of ammonium sulphate.

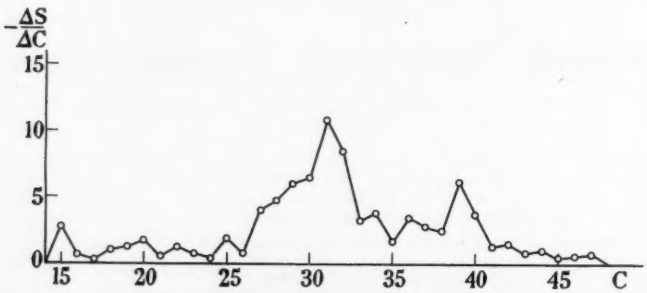

FIG. 4.—The derived solubility curve of a fraction extracted from isolated myofibrils of a dystrophic rabbit. The extract was obtained and treated by the same method as the extract of Fig. 3. A slight precipitation between 14 per cent and 26 per cent saturation of ammonium sulphate usually occurs in the fractions obtained from myofibrils of dystrophic rabbits : the same occurs to a larger extent in the extracts of whole dystrophic muscle (Azzone and Aloisi, 1958).

seemed to correspond to those of myosin and actomyosin ; the faster peak was much bigger than the other. That the faster peak was in fact actomyosin is supported by the following experiment. The protein precipitated by 0·3 M KCl was dialysed (24 hr.) against 0·10 M KCl containing 0·05 M K phosphate (pH 7·2) 0·02 M Na pyrophosphate, 0·002 M $MgCl_2$ and HCl to bring the final pH to 7.

TABLE.—*Properties of the KCl-pyrophosphate-$MgCl_2$ Extracts of Isolated Myofibrils from Normal (N) and Dystrophic (D) Rabbits*

Expt. No.	Protein precipitated at 35 per cent saturation $(NH_4)_2SO_4$ (as per cent of total protein)	Protein precipitated at 0·3 μ	Zη	Sensitivity to ATP
56N	22·6	15·4	0·20	10·4
59N	28·2	18·2	0·22	10·1
62N	35·6	24·7	0·21	17·6
66N	38·2	17·9	0·22	20·7
60D	62·5	46·6	0·27	36·5
61D	40·1	35·5	0·23	22·1
65D	53·0	46·0	0·28	40·8
69D	71·5	60·5	0·30	41·4

Under these conditions only one major peak was shown by electrophoresis analysis and a smaller one of much higher mobility, of the same order as that reported for actin (Fig. 5 and 6): apparently actomyosin had split into myosin and actin. No appreciable difference was detected in the samples obtained from normal and dystrophic rabbits.

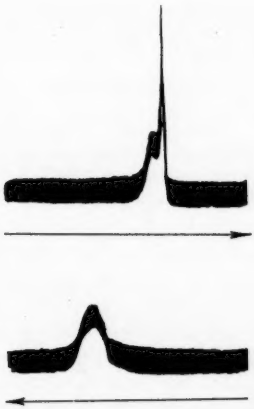

FIG. 5.—Electrophoresis diagram of a fraction precipitated by 0·3 M KCl from an extract of myofibrils of a dystrophic rabbit. Electrophoresis conditions: 0·26 M KCl, 0·05 M K phosphate, pH 6·9. After 805 min. The upper is the ascending boundary.

The acid-labile phosphorus content of myofibrils isolated from normal rabbits was about 120γ/g. dry weight (average of 5 preparations — 122γ/g.), a figure which is similar to that published by Perry. In myofibrils prepared from dystrophic rabbits the amount of acid-labile phosphorus was only slightly less (108γ/g. was the average of 4 preparations). The difference is of no statistical significance ($P > 0{\cdot}05$).

Both under normal and pathological conditions the proportion of ATP to ADP was approximately the same. The acid-labile phosphorus due to ATP was about one fifth of the whole acid-labile phosphorus.

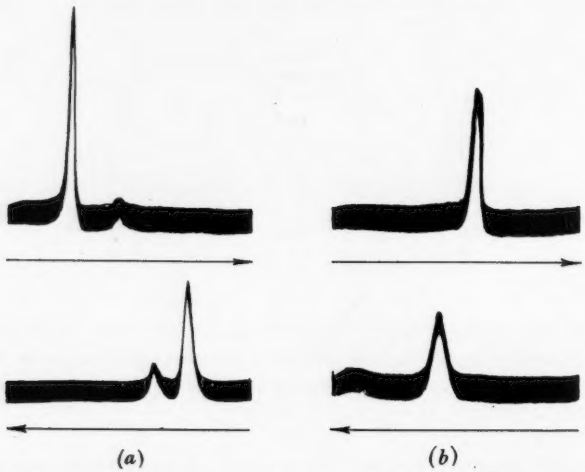

FIG. 6.—Electrophoresis diagram of the same fraction as in Fig. 5. Electrophoresis conditions: 10 M KCl, 0·05 M K phosphate ; 0·02 M Na pyrophosphate, 0·002 M $MgCl_2$, HCl to bring the final pH to 7. (*a*) After 240 min. (*b*) After 600 min. The upper is the ascending boundary.

DISCUSSION

From the evidence presented it appears that in the KCl-pyrophosphate + $MgCl_2$ extracts from isolated myofibrils of dystrophic rabbits the amount of actomyosin is much more than is the case with normal rabbits. The protein concentration in the extracts from dystrophic rabbits is not reduced : on the contrary it is slightly increased (on an average 68 per cent of the protein content of the myofibrils instead of 60 per cent) and the amount of " extra-protein " is not appreciably changed (about 12 per cent of the total protein extracted). Therefore the increase of actomyosin is not a relative one.

Our results may be explained by assuming that actin is more easily extracted from dystrophic myofibrils ; this fits in well with the fact that more protein is extracted from dystrophic myofibrils. There is not less myosin extracted, but rather more of it is complexed with actin in the extraction.

It seems likely that this is not a specific feature of vitamin E deficient muscle. Also in denervation atrophy it was reported (Fischer, 1950) that myosin is more appreciably reduced than actomyosin.

It has been suggested that the amount of actomyosin that can be extracted from muscle largely depends on the amount of ATP present (Szent-Györgyi, 1947). Moreover it has been shown that ATP plays a specific role in the polymerization of actin (Straub and Feuer, 1950). In our experiments most of the ATP was removed before the extraction, during the preparation of the myofibrils, and the amount of ATP which remains bound to the myofibrils after the prepar-

ation is about the same under normal and pathological conditions. It should therefore be assumed that the change in extractability of actin depends on some intrinsic change of this protein in dystrophic muscles.

This view is in agreement with the observations of Aloisi and coworkers (1951, 1952*b*, 1953) who showed that the properties of actin are appreciably changed in experimental dystrophy independently of the amount of ATP present in the muscle.

SUMMARY

The KCl-pyrophosphate-$MgCl_2$ extracts from isolated myofibrils have been investigated both in dystrophic and in normal rabbits after removing the " extra-protein " fraction.

From the electrophoretic analysis, viscosity measurements and the determination of the solubility curves in KCl and $(NH_4)_2SO_4$, evidence has been obtained that a normal amount of myosin is extracted from myofibrils of vitamin E deficient rabbits, but a larger proportion of this is bound with actin than is case with normal rabbits.

It appears that actin is more easily extracted from myofibrils of dystrophic rabbits on account of some intrinsic change.

The content of ATP bound to myofibrils is not appreciably changed in experimental dystrophy.

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THE EFFECT OF PREGNANCY ON THE RENAL RESPONSE TO RENIN

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THE injection of renin causes diuresis and natriuresis in the rabbit (Pickering and Prinzmetal, 1940; Brandt and Gruhn, 1948; Hughes-Jones, Pickering, Sanderson, Scarborough and Vandenbroucke, 1949; Rosenfeld, Sellers, Marmorston and Eliasch, 1954), and also in the rat (Addis, Barrett, Boyd and Ureen, 1949; Masson, Corcoran, and Page, 1950; Croxatto, Barnafi and Passi, 1952; Sellers, Smith, Goodman and Marmorston, 1951). This effect of renin appears to be mediated through hypertensin (Hughes-Jones *et al.*, 1949), and to be independent of its effect on blood pressure; a conclusion based on the finding that the increased excretion of sodium and water which follows the injection of renin occurs after the blood pressure has returned to normal. The difference in time relationship between pressor and diuretic effects is seen more clearly with hypertensin than with renin because of the very transient pressor response produced by hypertensin (Hughes-Jones *et al.*, 1949). Clearance studies suggest that increased sodium and water output in the urine result from direct inhibition of tubular reabsorption. The finding that the glomerular filtration rate actually falls during the pressor response, and is unaltered or diminished during the period when sodium and water excretion is increased, appears to rule out the possibility that it is due to increased glomerular filtration during the period of raised blood pressure. (Pickering and Prinzmetal, 1940; Hughes-Jones *et al.*, 1949; Brandt and Gruhn, 1948).

Since the diuretic-natriuretic response to renin appears to be independent of any rise in blood pressure, it was of interest to determine the effect of renin on the excretion of sodium and water by pregnant rats. The latter become relatively insensitive to the pressor action of renin (Mackaness and Dodson, 1957), but not to that of hypertensin (Dodson, 1957). An increased excretion of sodium and water in the pregnant animal would suggest that a defect in vascular reactivity might explain the diminished blood pressure response obtained with renin. Conversely, if the excretion of sodium and water was reduced in proportion to the fall in blood pressure response it would suggest that in pregnancy there was a defect in the enzymic reaction between renin and hypertensinogen, its substrate in the plasma.

MATERIALS AND METHODS

Animals

Female albino rats of Wistar origin, 10–16 weeks of age and weighing 150–200 g. were used. They were fed a diet of rat cubes which contained approximately 18 per cent crude protein and 0·5 per cent added sodium chloride. For each experiment, rats of similar ages were placed on clean litter in separate cages and were given water *ad libitum* but no food for 12–18 hr.

Renin

A lyophile-dried extract of pig kidney was prepared as described by Haas, Lamfrom and Goldblatt (1954). The intravenous injection of 1·5 mg. of this preparation produced an average blood pressure rise of 30 mm. Hg. in a group of 3 unanaesthetized rabbits (Pickering and Prinzmetal, 1938). This amount of renin is referred to as one rabbit unit (1·0 R.U.).

Measurement of kidney response

Each animal was given either water or 1 per cent NaCl solution by stomach tube; the choice depended upon whether or not a diuretic response to renin was being sought. In some experiments a second dose of water was given 1 hr. after the first. Following the first or second water load, renin was injected intraperitoneally in varying amounts in a volume of 0·5 ml. of saline. When the dose of renin varied, animals were alloted to dosage groups at random. The period of urine collection and other conditions of test were not uniform; details of procedure are therefore given together with the results. At each collection pressure was applied over the bladder to ensure complete emptying. If only one collection was required the bladder was emptied by forcing the animals to inhale ether vapour. The estimations of sodium were made with a flame photometer using an external standard.

RESULTS

The influence of Hydration and Dosage on the Renal Response to Renin

Before comparing the renal responses in normal and pregnant animals, several experiments were necessary in order to find conditions suitable to make the comparison.

In the first experiment, 32 rats were starved overnight. They were given 10 ml. of water by stomach tube. Immediately following this, saline or varying doses of renin were injected intraperitoneally, the animals being divided into 8 groups each of 4 rats according to dose. Urine was collected at the end of the 1st and 2nd hr. following the injections. In the second experiment, 42 rats were treated in the same way except that renin was injected at the end of the 1st hr. by which time water diuresis had become fully established in all animals.

The results of these experiments are illustrated in Figs. 1 and 2, in which the control values for animals injected with saline have been plotted along the ordinate axis. Fig. 1 shows that when renin and the water load were given together, intermediate doses of renin caused some increase in urine flow during the first hour. In the second hour, however, there was a progressive fall in urine volume as the dose of renin increased. Thus low doses of renin caused an increase in urine flow only while the rate of urine flow was low, and not during an established water diuresis. This is confirmed in the experiment shown in Fig. 2 in which renin was not injected until the animals had developed a full water diuresis. Here renin caused no increase in urine flow, its only effect being an antidiuresis at high dose levels.

The natriuretic response, on the other hand, was found to be independent of the state of hydration. During the first hour following the injection of renin, the sodium output increased linearly with the log-dose of renin injected over the range 0·125–2·0 R.U. This was seen (Fig. 1) when renin was given at the same time as the hydrating dose of water, and also when renin was injected into fully diuretic animals (Fig. 2). Doses in excess of 2·0 R.U. of renin caused a diminished output of sodium: these were the dose levels associated with an antidiuretic effect. Pickering and Prinzmetal (1940) attributed the antidiuresis seen during the pressor phase in rabbits to afferent glomerular arteriolar constriction with consequent fall in glomerular filtration rate. They also observed that the effect of renin on urine volume depended largely on the state of hydration of the animal.

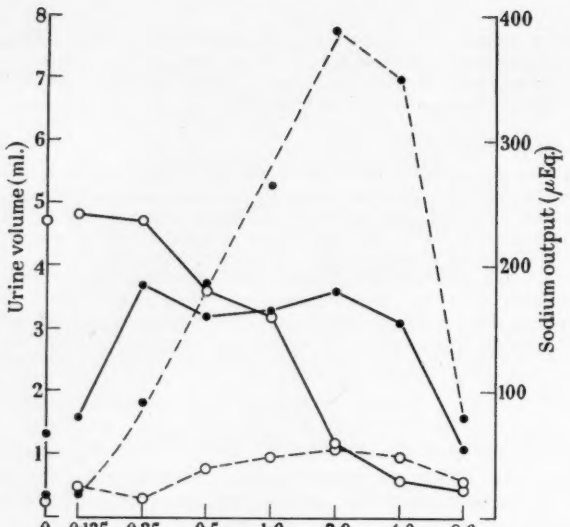

FIG. 1.—The mean output of water and sodium by groups of 4 rats during the first and second hour following a hydrating dose of water (10 ml.) by stomach tube, and the intraperitoneal injection of varying doses of renin. The results are plotted on semi-log co-ordinates. ●——● Urine volume first hour. ●- - -● Urine sodium first hour. ○——○ Urine volume second hour. ○- - - ○ Urine sodium second hour.

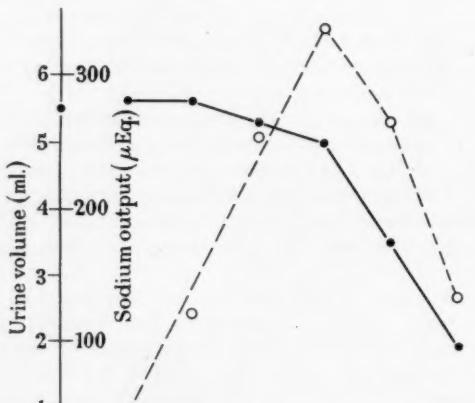

FIG. 2.—The mean output of sodium and water by groups of 6 rats during 1 hr. following varying doses of renin given by intraperitoneal injection at the height of a water diuresis. The results are plotted on semi-log co-ordinates. ●——● Urine volume. ○- - - -○ Urine sodium.

Similar experiments were performed in which 1 per cent NaCl solution was used to hydrate the animals. Under these conditions diuresis due to renin was not obscured by a coincident water diuresis.

The renal response to renin during pregnancy

In view of the previous results a procedure was adopted for comparing the renal responses to renin of pregnant and non-pregnant rats. The dose of renin used was 2·0 R.U., the largest dose possible without producing antidiuresis; the period of observation was restricted to the first one and a half hours after injection; saline instead of water was used to hydrate the animals so that the diuretic as well as the natriuretic response to renin could be observed.

At first an attempt was made to follow in the same animals a progressive change in the response to renin during the course of pregnancy. This had been done previously in respect to the pressor response to renin (Mackaness and Dodson, 1957). However, individual rats often showed increasing sensitivity to renin in successive tests (Mackaness and Dodson, 1957). The result was that a dose which caused diuresis and natriuresis in the first test was often sufficient to cause antidiuresis and interruption of the natriuretic response in the same animals in a subsequent test. To avoid this difficulty rats at varying stages of pregnancy were grouped and tested at the same time, together with a group of non-pregnant controls. The age of pregnancy in each rat was subsequently expressed in terms of the average foetal weight found at the time of test.

Twenty-two pregnant rats were used in the experiment, 24 days after they had been caged with males. Twenty-two unmated rats were used as controls. All animals were starved overnight on clean litter in separate cages. The following morning they were divided into 11 groups, each consisting of 2 pregnant and 2 non-pregnant animals.

Each rat was given 10 ml. of 1 per cent NaCl solution by stomach tube. Immediately following this, each pregnant rat and half the non-pregnant controls were injected with 2·0 R.U. of renin in 0·5 ml. of 0·9 per cent NaCl solution. The remaining non-pregnant rats were given an injection of 0·9 per cent NaCl solution without renin.

The bladder of each animal was emptied 90 min. after injection; the volumes of urine were measured and the cages and funnels were then washed down. The rats were killed subsequently, and the foetuses were recovered, counted and weighed.

Two rats littered overnight and a third during the experiment, and the results from these animals have not been included. The urine volume, sodium output and average foetal weight for each of the remaining 19 pregnant rats are given in Table I. The average foetal weight has been plotted against sodium output in Fig. 3. There is a highly significant correlation between the output of sodium and the average foetal weight. ($b = -192{\cdot}56 \pm 10{\cdot}9$, $P < 0{\cdot}001$). A similar relationship was found between urine volume and foetal weight. Although the foetal number varied between 5 and 12 calculation showed a better negative correlation between average foetal weight and renal response than between total fœtal weight and renal response. The results indicate that in response to renin the excretions of sodium and water by the kidney declined progressively as the age of pregnancy increased. The mean sodium output of 10 non-pregnant rats injected with renin was 1207 ± 46 (S.E.M.) μ equiv. in comparison with an output of 300 ± 10 (S.E.M.) μ equiv. in untreated animals. Thus the response of pregnant rats did not differ significantly from that of non-pregnant rats until pregnancy had advanced to a stage where the average foetal weight exceeded about 1·0 g.;

rats late in pregnancy excreted no more sodium in response to renin than did normal rats which had received no renin. Thus the effect of renin on the renal excretion of sodium and water showed the same progressive fall during pregnancy as was found in the case of the blood pressure response to renin (Mackaness and Dodson, 1957).

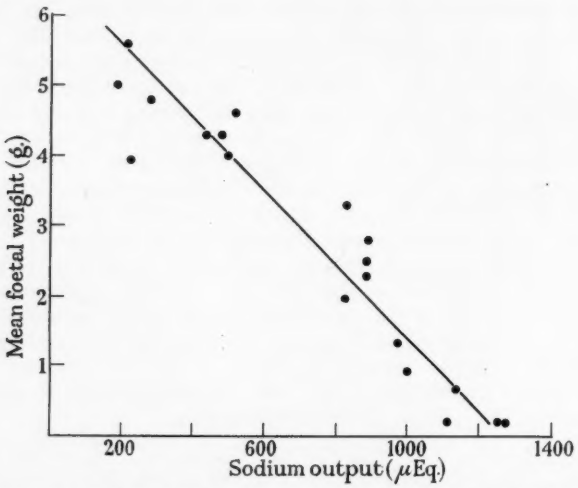

FIG. 3.—The regression of sodium output (S.O.) in μ equiv. on average foetal weight (F.W.) in g. for rats injected with renin at varying stages of pregnancy. The rats received 1 per cent NaCl solution by stomach tube (10 ml.) followed by an intraperitoneal injection of 2·0 R.U. of renin. The urine was collected for 1½ hr. after the injection. The regression line is given by the equation : S.O. = 733 − 192·56 (F.W. − 2·78).

TABLE I.—*The Urinary Output of Water and Sodium in Relation to the Age of Pregnancy in Rats Treated with 2·0 R.U. of Renin by Intraperitoneal Injection*

Average foetal weight (g.)	Urine volume (ml.)	Sodium output (μ equiv.)	Average foetal weight (g.)	Urine volume (ml.)	Sodium output (μ equiv.)
0·2	10·8	1270	4·0	4·5	495
0·2	5·3	1112	4·3	5·0	482
0·2	9·7	1288	4·3	2·2	435
0·7	8·7	1138	4·6	3·5	512
0·9	5·7	1000	4·8	1·8	273
1·3	4·9	975	5·0	1·4	195
2·0	4·9	825	5·6	0·8	210
2·3	5·8	887			
2·5	4·3	887	Untreated controls	2·6±0·1*	300±15
2·8	5·1	887			
3·3	4·4	825	Treated controls	7·7±0·4	1207±56
3·9	1·1	223			

* ± S.E.M.

The renal response of pregnant rats to varying doses of renin

An attempt was made to dissociate the vascular (antidiuretic) from the tubular (natriuretic) effects of renin, and to examine the effect of pregnancy on each of

them. This could be done by hydrating the animals with water and testing their responses to varying doses of renin over a prolonged period. A sufficient number of animals in an advanced stage of pregnancy could not be obtained at the one time to enable tests to be made simultaneously at each dose level of renin investigated. Instead, at each dose level groups of 6–9 pregnant rats were tested simultaneously with an equal number of non-pregnant controls.

The rats used were starved overnight, and after emptying the bladder each animal was given 10 ml. of water by mouth, followed by an intraperitoneal injection of a dose of renin in 0·5 ml. of 0·9 per cent NaCl solution. Urine was collected after 1, 2, 3 and 5 hr. The experiments were performed over several weeks under varying environmental conditions so that it was not possible to pool the data. However, comparisons can be made at any one dose level, since normal and pregnant groups were tested together. Pregnant animals were all within 3 days of littering; the numbers in their litters varied between 6 and 10.

The results are given in Table II and Fig. 4. They show, in confirmation of Hofmann, Knobil and Caton (1954), that pregnant rats excrete a water load exactly as normal animals do. During the water diuresis there was only slight loss of sodium in the urine of untreated animals of either group.

TABLE II.—*The Amount of Water (ml.) and Sodium (μ equiv.) Excreted by Hydrated Normal and Pregnant Rats during 5 hr. Following Intraperitoneal Injections of Varying Doses of Renin*

Dose of renin R.U.		Collection period: 1st hr. Water	1st hr. Sodium	2nd hr. Water	2nd hr. Sodium	3rd hr. Water	3rd hr. Sodium	4th and 5th hr. Water	4th and 5th hr. Sodium
0	N	5·0±0·6*	18±5	3·8±0·4	7±1	0·8±0·1	4±1	0·2±0·1	5±1
	P	6·1±0·8	37±9	2·9±0·2	8±2	0·8±0·2	4±1	0·4±0·2	5±2
1·0	N	4·0±0·5	258±21	3·4±0·4	54±9	1·4±0·2	7±1	1·1±0·2	4±1
	P	4·5±0·4	66±15	4·3±0·5	44±8	0·8±0·2	17±6	0·6±0·1	18±7
2·0	N	4·4±0·5	404±52	2·6±0·3	50±9	1·9±0·3	4±1	2·1±0·3	14±2
	P	4·2±0·4	68±6	5·2±0·2	70±8	1·2±0·1	45±5	1·1±0·1	39±6
4·0	N	4·1±0·7	400±32	1·3±0·3	54±10	1·0±0·2	15±1	2·1±0·3	9±1
	P	5·1±0·6	271±44	4·0±0·5	168±30	1·9±0·4	76±21	1·3±0·1	54±11
8·0	N	1·9±0·4	99±22	0·6±0·2	27±18	1·4±0·2	58±20	3·8±0·4	101±29
	P	3·8±0·4	254±39	3·5±0·3	190±19	2·5±0·3	75±16	2·2±0·2	104±22

N = Normal. P = Pregnant. * ± S.E.M.

As the dose of renin was raised from 1·0 to 8·0 R.U. normal animals showed a progressive impairment of water excretion. This contrasted with a progressive enchancement of water diuresis in the pregnant rats. The pattern of sodium excretion was different. As before, the sodium excreted by pregnant rats was less than that excreted by normals at low doses of renin; at higher doses, however, pregnant animals continued to excrete large quantities of sodium for several hours, so that their total output over 5 hr. was greater than that of normal rats. This difference between the groups was increased at the highest dose by the fall in sodium output

and accompanying antidiuresis which occurred in normal but not in pregnant animals.

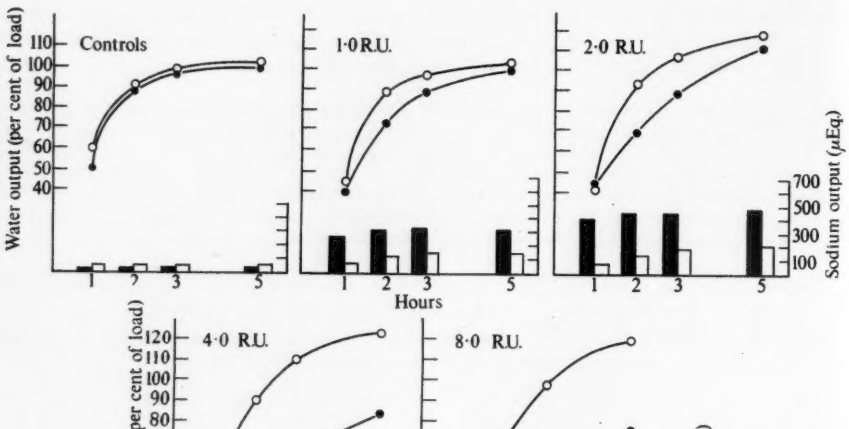

FIG. 4.—The water excretion curves and cumulative sodium output by normal and pregnant rats, hydrated with water and injected intraperitoneally with varying doses of renin. ●——● Water excretion by normal rats. ○——○ Water excretion by pregnant rats. ■ Sodium excreted by normal rats. □ Sodium excreted by pregnant rats.

DISCUSSION

Measurements of renal clearance (Braun-Menéndez, 1956) and serial angiograms of the renal circulation taken during a renin response (Daniel, Prichard and Ward-McQuaid, 1954) indicate that when antidiuresis occurs in response to renin it is due to interference with the intra-renal circulation. This leads to a reduction in renal blood flow and glomerular filtration rate, which may persist beyond the pressor phase of the renin response. A sufficient reduction in the volume of glomerular filtrate would interfere with a water diuresis by limiting the volume of water entering the distal convoluted tubules where facultative control over water excretion occurs. In the same way, the natriuretic effect of renin, which is thought to be due to inhibition of sodium reabsorption by the distal tubules, cannot occur unless the volume of glomerular filtrate is large enough to ensure that sodium is reaching the distal tubules. With these considerations in mind, it is possible to interpret the present experimental results.

When conditions were chosen so that the period of observation was confined to the first hour and a half, and the dose of renin was insufficient to cause anti-

diuresis, the renal response of late pregnant rats was found to be much less than that of non-pregnant controls. Moreover, the response fell progressively as pregnancy advanced. Thus the renal response and the pressor response are both diminished in pregnant rats. It is concluded that pregnancy causes an overall depression of the renin-hypertensin reaction, thus accounting for its interference with such diverse effects as the vascular (pressor) and renal tubular (natriuretic) actions of renin.

It might appear paradoxical that by increasing the dose of renin the natriuretic response of pregnant rats was made to exceed that of normal animals. The pregnant rats, however, were relatively insensitive to renin, and suffered no impairment of their renal circulation even from the largest dose of renin given. This is shown in Fig. 4 by the steepness of the excretion curves in pregnant rats. In the absence of any impairment of the renal circulation a large dose of renin would continue for a long time to exert an effect on salt and water output.

A dose of 4·0 R.U. of renin has no significant effect upon the blood pressure of late pregnant rats (Mackaness and Dodson, 1957). Nonetheless Fig. 4 shows that this dose produced a considerable natriuresis in pregnant rats. This seems to provide additional evidence that the diuretic and natriuretic effects of renin do not depend upon a rise in blood pressure.

Water diuresis is brought about by the withdrawal of antidiuretic hormone. This results in a maximum rejection of water by the distal convoluted tubules. The inability of renin to increase urine flow during water diuresis suggests that renin acts on that part of the renal tubule which is controlled by the antidiuretic hormone (ADH), and is therefore already impermeable to water at the height of a water diuresis. This is consistent with the finding of Croxatto, Barnafi, Lastra, Parra and Vera (1953) that renin antagonises the diuretic effect of ADH. The failure to increase urine flow during water diuresis and the antagonism of ADH support the evidence already given that the diuretic-natriuretic effect of renin is a tubular one.

Although the vascular and renal responses to renin appear to be due to different and independent effects of renin it has been found from the present experiments that pregnancy causes a comparable depression of both. It is concluded that the altered response in pregnancy is not primarily a matter of diminished vascular reactivity, but probably a disorder of the renin-hypertensin reaction itself.

SUMMARY

Pregnant rats show little or no rise in blood pressure when injected with doses of renin which are pressor in normal animals. A study was made therefore, of the effect of pregnancy on the diuretic and natriuretic responses to renin, since these occur independently of any rise in blood pressure. The diuretic effect of renin, which could be demonstrated only at low rates of urine flow, was found to be diminished in pregnant rats. The natriuretic effect on the other hand was unaffected by the state of hydration, but it too, was diminished during pregnancy. The magnitude of the response in both cases fell progressively as pregnancy advanced ; a significant negative correlation was found between water or sodium output and the average foetal weight at the time of test.

High doses of renin were antidiuretic, probably because they interferred with renal blood flow. This effect of large doses of renin was also depressed by preg-

nancy. Thus the insensitivity of pregnant rats to renin involves both vascular (pressor and antidiuretic) and renal tubular (natriuretic) effects. Presumably therefore it cannot be due primarily to diminished vascular reactivity, but must involve the renin-hypertensin reaction itself.

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OBSERVATIONS ON THE INTERACTION OF POLIOVIRUS AND HOST CELLS *IN VITRO*

III. THE EFFECT OF SOME BACTERIAL METABOLITES AND ENDOTOXINS

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THE multiplication of poliovirus in tissue culture can be influenced by a number of different substances. Thus, Eagle and Habel (1956) showed that only minimal amounts of virus were formed in HeLa cell cultures if the medium did not contain glucose or glutamine, while Rappaport (1956) found that good virus synthesis of poliovirus occurred in monkey kidney in a synthetic medium containing only cysteine, isoleucine and D-ribose in a buffered salt glucose medium. Likar and Wilson (1959) demonstrated that higher titres of poliovirus could be obtained when insulin was added to the tissue culture media, and in the present communication it is shown that some substances derived from human faecal bacteria can diminish the amount of poliovirus produced in monkey kidney monolayers.

These experiments were done to try and explain why some individuals who were devoid of antibody failed to excrete demonstrable virus after feeding with an oral poliovirus vaccine (Dane, Dick, Connolly, Fisher and McKeown, 1957). Although our experiments cannot exactly imitate the conditions under which epithelial cells of the human intestines are subjected to infection with poliovirus, the results suggest that bacterial intestinal flora might play a role in the dynamics of viral multiplication in the human gut.

MATERIALS AND METHODS

The methods used have been described (Likar and Wilson, 1958). Second-passage monkey kidney cells grown in monolayers for 3 days at 37° were treated with : sterile crude filtrates of the cultures of faecal bacteria grown under aerobic and anaerobic conditions ; endotoxins derived from the aerobic and anaerobic bacterial cultures isolated from one of the specimens of faeces ; a preparation of endotoxin of *Salmonella typhimurium* kindly supplied by Dr. A. W. Gledhill.

The specimens of faeces were obtained from three individuals and were labelled A, B and C. The filtrates were prepared by plating a suspension of the faeces to be tested on Maconkey, Leifson and on blood agar plates which were incubated aerobically and anaerobically at 37° for 4 days. A mass inoculum from the culture on each plate was then transferred to nutrient broth and these subcultures were grown aerobically and anaerobically for a further period of 4 days. The broth subcultures of each faecal specimen were pooled and centrifuged at 2,500 r.p.m. for 20 min. and the supernatant fluid was then sterilised by passage through a sintered glass filter (G5) and stored at — 70°.

From one of the specimens of faeces (C) pure cultures of *Bacterium coli* (4 strains), *Streptococcus faecalis* (2 strains) and one strain of a diphtheroid were isolated. Endotoxins were

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prepared from these cultures by centrifugation and resuspension of the bacterial cells in distilled water, followed by repeated thawing and freezing at — 70° and centrifugation at 2,500 r.p.m. for 20 min. The supernate was then sterilised by passing the suspension through a sintered glass filter (G5) and stored at — 70°.

Various dilutions of these filtrates were added to the fluid medium of 3 days old tissue cultures. For the next 2 days the culture fluids were removed daily and replaced by fresh medium containing the same dilution of the filtrates. Three days after the addition of filtrates or in some experiments in a shorter period the cultures were infected with type 1

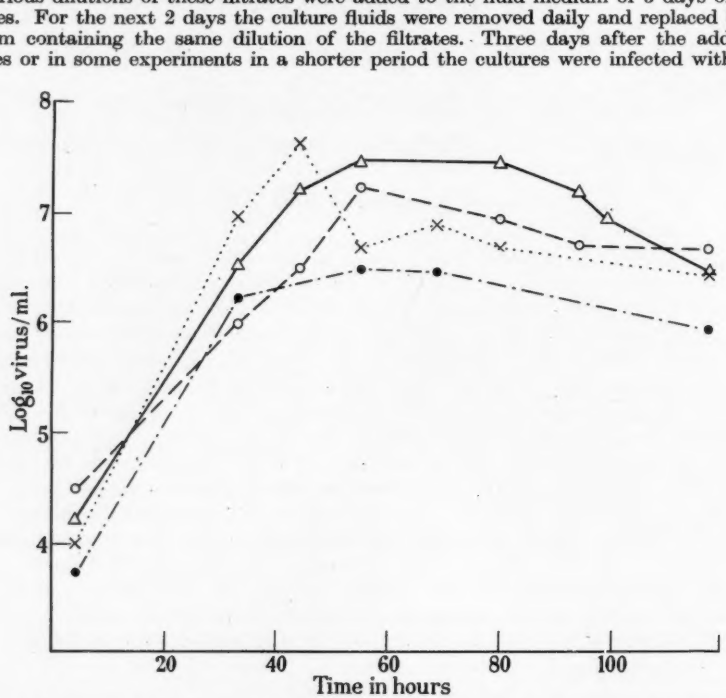

FIG. 1.—The amount of poliovirus present in supernatant fluid of cultures at given intervals of time treated with filtrates of the cultures of faecal bacteria diluted 1 : 10 from 3 individuals: A (○- - - -○), B (×. . . . . ×) and C (●-·-·-·-●) and in supernatant fluid of cultures incubated without the addition of filtrates before being infected with poliovirus (△———△).

poliovirus (Mahoney strain) giving a final virus concentration of 10^3 to 10^4 TCD_{50}. Fresh bacterial filtrate was not added after the tubes with tissue culture were infected. Monkey kidney monolayers incubated without the addition of filtrates before being infected with poliovirus were used as controls for each experiment.

The fluids of 4 to 6 filtrate treated cultures and of control cultures were harvested at intervals of approximately 10 hours (see Fig. 1), pooled and titrated after storage at — 20° for 2 to 8 weeks. In any one experiment all the samples of culture fluid were titrated at the same time and 4 to 6 tubes were used for each dilution tested.

RESULTS

Filtrates of the cultures of faecal bacteria

It was found that undiluted crude filtrates of the bacteria of the three faecal samples were toxic for the tissue culture cells; at a dilution of 1 : 2 there was no

visible toxic effect. While a 1 : 500 dilution had no effect on the multiplication of poliovirus, effects on the virus production were noticed at a 1 : 10 dilution as shown in the experiment recorded in Fig. 1, which records the results of one experiment.

On repetition, the results were reproducible. From Fig. 1 it may be seen that, apart from a slight peak in the titre of virus at 44 hr. in the cultures treated with filtrate B, the virus liberated by all cultures treated with the filtrates was less than that in controls, however the difference was significant only in the cultures

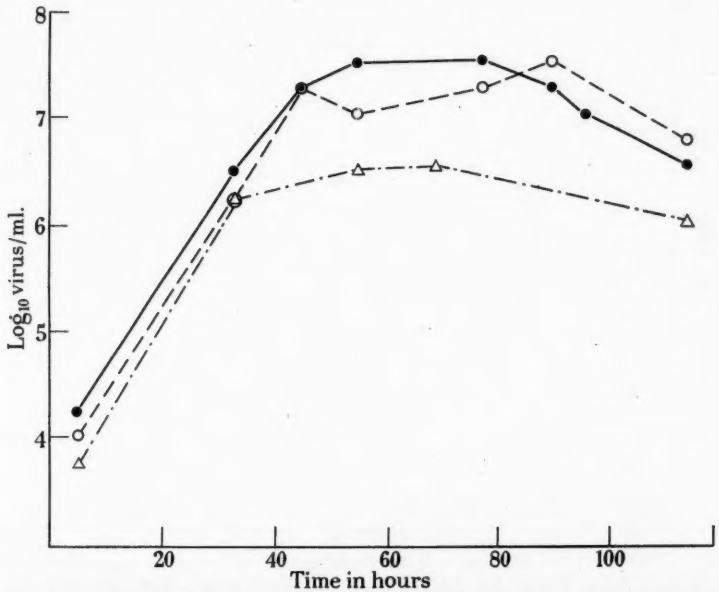

FIG. 2.—The amount of polivirus present in supernatant fluid of cultures at given intervals of time treated with the filtrate of the cultures of faecal bacteria from individual C in dilution 1 : 10 (△-·-·-·△), 1 : 1000 (○- - - -○) and in supernatant fluid of cultures incubated without the addition of the filtrate (●———●).

treated with filtrate C. The probability that differences as great or greater between controls and the cultures treated with filtrates A, B or C could arise by chance were $> 0{\cdot}1$, $> 0{\cdot}1$ and $< 0{\cdot}05$ respectively.

The effects of undiluted filtrates of specimens A and B were also tested. It was found, somewhat surprisingly, that the undiluted A filtrate, although toxic to the cells, did not significantly affect the virus production of the monolayer, while the undiluted B filtrate had an inhibitory effect similar to that produced by a 1 : 10 dilution. An experiment was done with serial dilutions of filtrate C which had most effect at a 1 : 10 dilution. It was found that there was a significant reduction of virus multiplication at dilutions up to 1 : 100 of this filtrate, but this was absent at 1 : 1,000.

Endotoxins from individual cultures

An attempt was made to determine whether the inhibitory effect was due to some substance in the endotoxin preparations from pure cultures of each of the several bacterial species isolated from specimen " C ". Each of these preparations was added to tissue cultures so as to give final dilutions of 1 : 10, 1 : 50 and 1 : 100. The results showed that the extracts from one strain of a diphtheroid and from 2 strains of *Str. faecalis* and from one strain of *Bact. coli* had no significant effect on the amount of poliovirus produced. On the other hand, the endotoxin prepara-

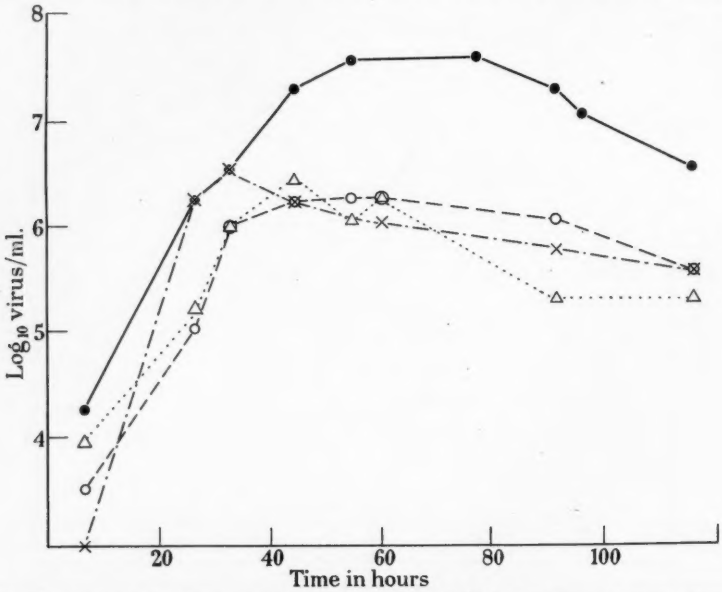

FIG. 3.—The amount of poliovirus present in supernatant fluid of cultures at given intervals of time treated with endotoxin preparations diluted 1 : 50 from 3 strains of *Bact. coli* isolated from individual C : strain 1 (▽.....△), strain 2 (○– – –○), strain 3 (×–·–·–·–×) and in supernatant fluid of cultures incubated without the additon of endotoxins (●———●).

tions from the other three strains of *Bact. coli* tested had an inhibitory effect at a dilution of 1 : 50 as illustrated in Fig. 3.

The probabilities that the difference observed with each of the filtrates as compared with the control was due to chance are $< 0{\cdot}01$ for the three *Bact. coli* endotoxin preparations.

It can be concluded that 3 of the strains of *Bact. coli* possess a principle which has an effect on the amount of poliovirus produced in tissue culture similar to that produced by the crude filtrate of the faecal flora of specimen C.

Endotoxin from Salmonella typhimurium

Monkey kidney tissue cultures were treated by the addition of glucolipid polysaccharide endotoxin diluted 1 : 10 to 1 : 5,000. The effect on the growth

of poliovirus was similar to that previously obtained with the crude bacterial endotoxins of *Bact. coli* but it was much more pronounced, viral multiplication in some experiments being entirely suppressed.

It was found that the effect of the endotoxin on the cells was influenced by two main factors : the duration of treatment with endotoxin and its concentration. Fig. 4 illustrates the effect of the time factor ; it shows that the growth of virus in cells treated with endotoxin for 3 days before infection with virus was completely suppressed and if endotoxin and virus were added simultaneously there was marked inhibition of virus production but not complete suppression.

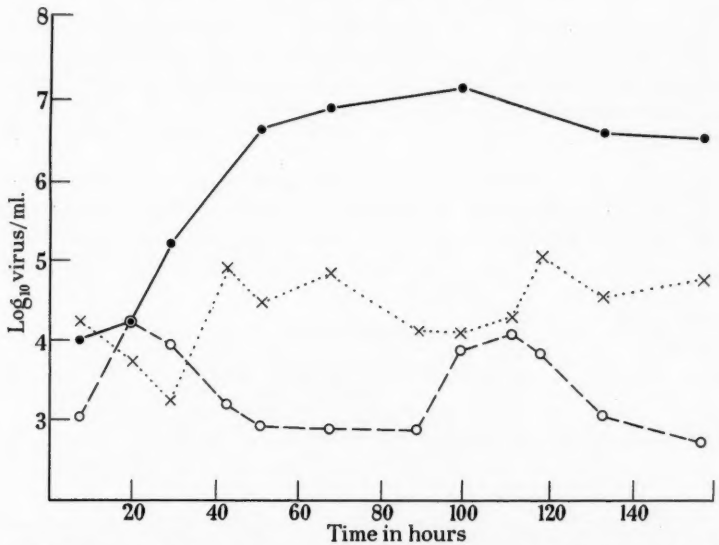

FIG. 4.—The amount of poliovirus in supernatant fluid of cultures at given intervals of time treated with endotoxin from *Salm. typhimurium* diluted 1 : 10 for a period of three days (○- - - -○) in cultures to which endotoxin and poliovirus were added simultaneously (×..... ×), and in cultures incubated without the addition of endotoxin (●———●).

In Fig. 5 the effect of dilution of endotoxin on the production of virus is illustrated. The dilutions used ranged from 1 : 10 to 1 : 5,000 and after 3 days treatment with endotoxin the cultures were infected with poliovirus. It is shown that under these conditions a dilution of 1 : 10 of endotoxin completely prevented normal multiplication of virus. Despite this, the cytopathogenic changes observed in these cultures after infection with virus corresponded exactly to those seen in control cultures.

The effect of the substances tested on the free poliovirus in tissue culture fluids was measured. Tissue culture fluid containing 10^4 TCD_{50} of type 1 poliovirus was incubated with the bacterial filtrates and the amount of the virus present was titrated at 24 hr. intervals. It was found that the diminution of the amount of the virus in the fluids with bacterial filtrates was not greater than in

control tissue culture fluids which did not contain bacterial filtrates where there was a gradual thermal inactivation of the virus at 37°.

DISCUSSION

In the experiments described above, it has been shown that various bacterial products which occur naturally in large quantities in human faeces reduce the amount of poliovirus produced in tissue culture fluids of monkey kidney monolayers.

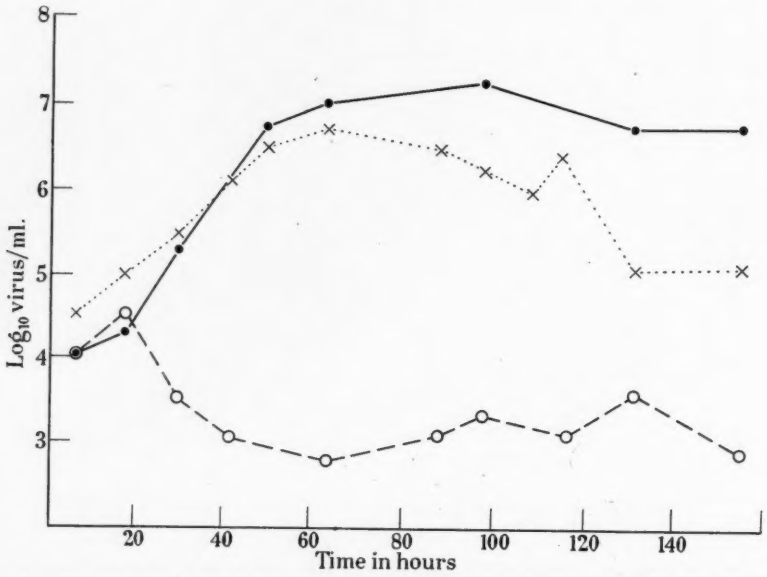

FIG. 5.—The amount of poliovirus in supernatant fluid of cultures at given intervals of time treated with endotoxin from *Salm. typhimurium* diluted 1 : 10 (○- - - -○), 1 : 500 (×.....×) and in supernatant fluid of cultures incubated without the addition of endotoxin (●——●).

It could be argued that the effect we have observed is quite simply due to a "toxic" effect of the bacterial filtrates on the cells or due to inactivation of the free virus. When undiluted filtrates were used which produced "toxic" effects on the cells, the titres of poliovirus in the tissue culture fluids were not different from titres obtained in experiments where no "toxic" effect was observed. Indeed in several experiments where the "toxic" effect was so great as to produce rounding of the cells and their separation from the glass the resulting virus produced was not significantly different from that in tubes with cells in monolayers. We have also observed on numerous occasions a suppression of virus production in tissue cultures treated with bacterial filtrates. In these instances the filtrates have produced no visible change in the cultures and the cytopathogenic effects seen after infection with virus have not differed from those observed in control

cultures. We do not therefore think that the inhibitory effect which we have observed is due to a crude " toxic " effect on the cells of the monolayers.

It was shown experimentally that the filtrates tested did not have a direct inactivating effect on the free virus of the tissue culture fluids. Furthermore, in none of the experiments could we find evidence that the attachment of virus to the cells was prevented.

It would appear, therefore, that the endotoxin filtrates affect virus multiplication within the cells.

SUMMARY

Sterile crude filtrates of the normal bacterial flora of 2 out of 3 persons tested suppressed the growth of poliovirus in monkey kidney tissue cultures. The active principle was found to be present in 3 out of 4 strains of *Bact. coli* isolated from one of these persons. Partly purified endotoxin of *Salm. typhymurium* produced the same effect to a much higher degree.

Our thanks are due to Professor G. W. A. Dick for his help and advice. We also wish to thank Dr. D. S. Dane, Department of Microbiology, Dr. E. A. Cheeseman, Department of Social and Preventative Medicine, The Queen's University of Belfast, Mr. D. Mehaffey and Mr. F. Burns, F.I.M.L.T.

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CHICKENPOX AND HERPES ZOSTER

1. COMPLEMENT FIXATION STUDIES

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THE close relationship between chickenpox and herpes zoster was first recorded by von Bókay in 1892 (von Bókay, 1909) and indeed the occurrence of chickenpox in child contacts of zoster cases is by no means uncommon (Simpson, 1954). In spite of the experimental production of chickenpox in children inoculated with zoster vesicle fluid (Kundratitz, 1925; Bruusgaard, 1932) the difference in clinical picture, infectiousness, age and seasonal incidence, has led to some hesitation in acceptance of the view that the 2 diseases are caused by the same virus. Experimental work with virus from these diseases has been restricted because of the specificity of the virus for the human host. However, when in 1952 Weller and Stoddard showed that virus from both diseases could be propagated in tissue cultures of human cells this difficulty was overcome. In the present work use has been made of this and other new techniques in a laboratory study of the relationship of viruses isolated from chickenpox and herpes zoster cases. In this paper the results of studies on the antigenic relationship determined by the complement-fixation technique are given, while later papers will deal with the results obtained by precipitation tests using the Ouchterlony technique, the behaviour of strains in tissue culture and the results of neutralization tests.

In the past, serological studies on zoster and chickenpox have been made by various workers using the complement-fixation technique. Antigens prepared from crusts or vesicle fluid have been tested against convalescent sera from the 2 diseases. Conflicting results have been obtained, perhaps because of differences in technique. Netter and Urbain (1926) used mostly extracts of crusts as antigen in the study of sera from 100 cases of zoster and 24 sera from cases of chickenpox. Antigen and serum were kept constant in amount and varying doses of complement were used. They found that approximately 90 per cent of convalescent sera from either disease gave positive results with antigens from both. Lauda and Silberstern (1925) on the other hand found that 25 sera from zoster cases gave negative results with chickenpox crust antigen. Brain (1933) strongly recommended the use of diluted vesicle fluid as antigen since crust extracts were frequently highly anticomplementary. In tests of sera from 44 cases of zoster and 17 cases of chickenpox he found that both kinds of sera gave fixation with vesicle fluid from either disease. Accurate comparison of his cross fixation tests, however, is not easy, as apparently only a single serum dilution was used. Thomsen (1934) failed to obtain fixation of complement when zoster sera were tested with varicella crust

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antigen, or when varicella sera were tested with zoster crust antigen. On the other hand, Hasskó, Vamos and Thoroczkay (1938), who used varicella crust and zoster crust or vesicle fluid antigen, found that cross reactions did occur but they were weaker than the reactions obtained when sera were tested against homologous antigen. Recently, Weller has used, as antigen, tissue culture fluids concentrated by ultrafiltration (Weller, 1958; Weller and Witton, 1958). The sera from both diseases gave similar results with these tissue culture antigens produced by virus from either chickenpox or zoster cases.

In the results recorded below crusts were not used as a source of antigen for, as Brain found, extracts made from them were highly anticomplementary and weak in specific activity. Diluted vesicle fluids or tissue culture fluids have been used as antigens and sera have been tested in doubling dilutions using a constant dose of complement.

MATERIALS AND METHODS

Antigens.—Vesicle fluids collected from chickenpox and zoster patients by means of finely drawn Pasteur pipettes were diluted in fluid often containing 10 per cent horse serum and then stored at — 70° in hard-glass tubes. Vesicle fluids were tested for antigenic potency before use and details of this are given below. Tissue culture fluids to be used as antigen were obtained as follows:— human amnion cells, grown in 6 oz. flat-sided bottles (soft glass; U.G.B.) or 6 × $\frac{5}{8}$ in. rimless test tubes (Pyrex) by the technique of Zitcer, Fogh and Dunnebacke (1955) were infected by inoculation with chickenpox or zoster vesicle fluids or with other infected amnion cells. After incubation at 37° for varying periods the nutrient medium from such infected cultures was removed and stored at — 70°; in some cases fresh medium was added and again removed after a variable time and stored, this process being repeated perhaps 3 or 4 times.

Sera.—These were obtained from chickenpox and zoster cases usually within the first few days of the onset of the rash and again about 3 weeks later. From a group of chickenpox patients, specimens of serum were collected at intervals of a few days up to 25 days after the onset of the rash. All sera were stored at — 20° in hard-glass tubes. On the day of the test appropriate amounts were inactivated at 56°–58° for 20 min. and suitable serial dilutions made in normal physiological saline.

Complement.—Azide-preserved complement stored at 4° was used. Before each experiment the complement was titrated with 2 per cent sensitized sheep red cells, and a titre of 1/60 to 1/70 was considered satisfactory. Two and a half doses of complement were used in the tests, varying doses being employed in the controls.

Sensitized sheep red cells.—Sheep red cells were used soon after collection or after storage in Alsever's solution at 4°.

The cells were washed 3 times and suspended in saline to make a 4 per cent suspension. Haemolysin (Burroughs Wellcome) stored at 4° was diluted to a strength of 1/250 (4 MHD) in normal saline and then equal quantities of this dilution and 4 per cent red cell suspension were well mixed and allowed to stand at room temperature for 30 min. to produce a 2 per cent suspension of sensitized cells ready for use.

Controls.—In all experiments in which convalescent sera of either chickenpox or zoster cases were used, an acute phase serum was included as a negative control. In all tests the antigen was examined for anticomplementary activity either against different doses of complement ($2\frac{1}{2}$, 2, $1\frac{1}{2}$, and 1 MHD) or in a single tube against $1\frac{1}{2}$ MHD. Similar serum controls were also made, the first 2 or 3 dilutions of each serum being tested with $1\frac{1}{2}$ MHD of complement only, $2\frac{1}{2}$ MHD being used in the test proper.

Procedure.—The tests were carried out in $3\frac{1}{2} \times \frac{1}{2}$ in. tubes. To each tube, containing 0·1 ml. of serum dilution and 0·1 ml. of complement, 0·1 ml. of antigen was added. Racks were shaken well to facilitate mixing and then left overnight at 4°. Next morning they were again shaken and left at room temperature for 30 min. before the addition of 0·2 ml. of sensitized sheep red cell suspension to each tube. The tubes were incubated in a water-bath at 37° for 30 min. Readings were made after they had stood for a further 1–2 hr. at room temperature to allow the non-haemolysed cells to settle.

RESULTS

Tests with Zoster Vesicle Fluid and Chickenpox and Zoster Sera

Preliminary titration of zoster vesicle fluids

Such fluids varied in antigenic potency and the results of the titration of one (ZPA.) which was used in most of the work is shown in Table I. This fluid, collected from a patient on the 5th day of the eruption, was diluted with an equal volume of tissue culture medium containing 10 per cent horse serum and stored for 4 months at −70°. Dilutions of this fluid were made in saline and tested against a convalescent zoster serum, collected 25 days after onset of the rash, and an acute chickenpox serum, collected on the first day of the rash.

It may be seen from Table I that this fluid had quite a high content of specific antigen; indeed it had a higher antigen titre than most of those examined. It was used in a dilution of 1/50 in routine tests of other sera. At this dilution it showed good serological reactivity and little anticomplementary effect. Other zoster vesicle fluids were tested in the same way and when suitable used in that dilution which gave the same titre as a 1/50 dilution of fluid ZPA. with the convalescent serum shown in Table I. This standard was adopted to conserve supplies of fluids which were only obtainable in small quantities. Less dilute vesicle fluids gave higher serum titres than those obtained with a 1/50 dilution of ZPA., and in tests made to determine the time of maximum antibody levels in chickenpox convalescents a stronger antigen was used.

TABLE I.—*Titration of Zoster Vesicle Fluid* ZPA.

Antigen dilutions	Serum dilutions: Convalescent zoster serum 1/4	1/8	1/16	1/32	1/64	1/128	Acute chickenpox serum 1/4	1/8	1/16	Antigen control (1½ MHD of complement)
1/10	++++	++++	++++	++++	+++±	+++	+++	+++	+++	++
1/20	++++	++++	++++	++++	++	+	+	+	+	±
1/40	++++	++++	++++	++++	+	−	−	−	−	−
1/80	++++	++++	++++	++++	±	..	..	..	..	..
1/160	++++	++++	+++	+	..	..	..	..	..	..
1/320	−	−	−	−	..	..	..	..	..	..
Serum controls (1½ MHD complement)	−	−	−	..	..	..	−	−	−	..

− indicates complete haemolysis.
++++ ,, no haemolysis.
+++, ++, +, ± increasing degrees of haemolysis.

Titration of sera using zoster vesicle fluid antigen

Table II shows the results of tests with representative samples of paired sera from zoster and chickenpox patients against the standard vesicle fluid antigen ZPA. Serum and antigen controls were all negative and are not recorded. Figs. 1 and 2 record the results of all zoster and chickenpox sera tested against zoster antigen and the stage of illness at which the sera were collected.

TABLE II.—*Complement-fixation Test with Vesicle Fluid Antigen* ZPA. *and Zoster and Chickenpox Sera*

Patient	Age (yrs.)	Duration of rash when serum taken (days)	Zoster sera Serum dilutions 1/4	1/8	1/16	1/32	1/64
A. B—	58	4	±	−	−	−	−
		9	++++	++++	+++	+	±
		35	++++	++±	±	−	−
L. O—	69	3	±	−	−	−	−
		23	++++	++±	−	−	−
S. A—	28	4	−	−	−	−	−
		25	++++	++	±	−	−
C. B—	38	2	+	−	−	−	−
		23	++++	++++	+	±	−
P. M—	26	4	+++±	±	−	−	−
		26	++++	++++	+++±	++	+
J. N—	28	5	∓	−	−	−	−
		16	++++	++++	+++	±	∓
		26	++++	+++±	++	±	−
J. P—	67	5	++++	++++	+++	++	0
		26	++++	+++	++	+	∓
L. W—	8	7	+++	+	±	−	−
		28	++++	++++	++	±	−
		Chickenpox sera					
M. A—	2	3	−	−	−	−	−
		19	++++	++	∓	−	−
M. G—	3	4	−	−	−	−	−
		18	++++	+	∓	−	−
P. D—	33	4	−	−	−	−	−
		25	++++	+++	±	−	−
A. C—	6	3	−	−	−	−	−
		24	++++	++	±	−	−
A. P—	5	3	−	−	−	−	−
		24	++++	+	±	−	−
S. D—	14	2	−	−	−	−	..
		23	±	∓	−	−	−
S. H—	7	2	−	−	−	−	..
		27	++	±	−	−	−
P. O—	7	2	−	−	−	−	..
		24	+	∓	−	−	−

0 = not tested.

It may be seen from Table II and Figs. 1 and 2 that, with zoster vesicle fluid, the convalescent zoster sera generally gave higher titres than did the convalescent chickenpox sera. This might suggest only a partial sharing of antigens by 2 different viruses, but the results with chickenpox vesicle fluid recorded later (Table IV) do not support this suggestion. There is an indication from the results in Table II that the titre of complement-fixing antibody may be declining by the 35th day from the onset of the rash (serum A.B.) or even after 26 days (sera J.N. and J.P.) As repeated blood samples were not obtained from zoster patients there is no clear evidence on this point. However, multiple samples of serum were collected from 7 cases of chickenpox in a boarding school outbreak. These gave weak titres with standard zoster antigen ZPA. and were tested against a stronger antigen (ZNE.) to follow the changes in antibody levels. The results of tests

of sera from 3 of the boys are shown in Table III and indicate that complement-fixing antibody is at its highest in the 2nd or 3rd week of illness.

The results of serum samples taken in the first few days after the onset of the rash indicated that the antibody response occurred earlier in zoster than in chickenpox.

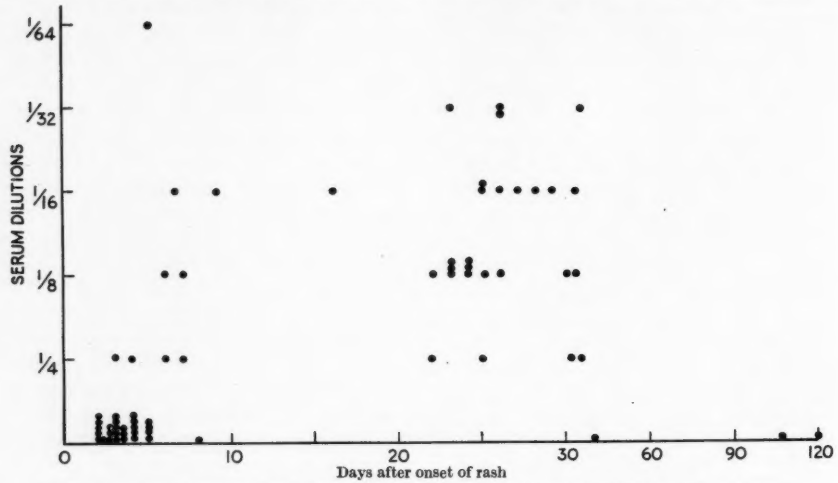

FIG. 1.—Complement-fixing titres of zoster sera tested with standard zoster antigen.

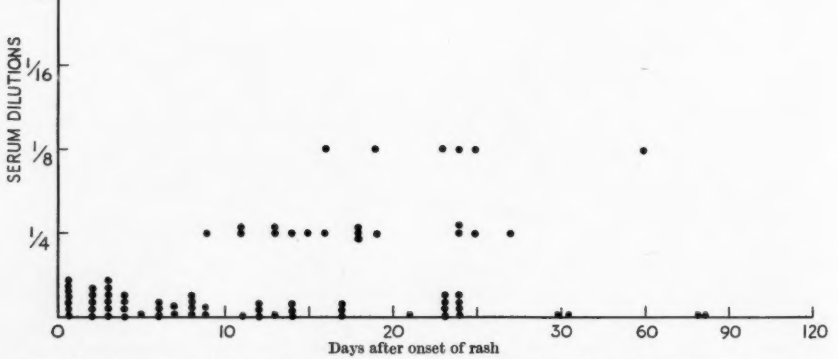

FIG. 2.—Complement-fixing titres of chickenpox sera tested with standard zoster antigen.

Several zoster sera taken during the first 8 days gave positive results with standard antigen while all the chickenpox sera collected at this time were negative (Figs. 1 and 2).

There was no correlation between the age of the patient and the degree of antibody response in zoster cases; equally high titres were obtained from young and elderly patients.

TABLE III.—*Sera from 3 Chickenpox Patients Tested with Zoster Vesicle Fluid ZNE.*

Days after rash	Serum dilutions: Patient B. 1/4	Patient B. 1/8	Patient B. 1/16	Patient Wi. 1/4	Patient Wi. 1/8	Patient Wi. 1/16	Patient Wh. 1/4	Patient Wh. 1/8	Patient Wh. 1/16
1	−	−	−	−	−	−	−	−	−
9	++++	++±	−	..	..	..	++	−	−
12	++++	+++±	−	+	−	−	+++	−	−
14	++++	+	−	+++±	−	−	+++	±	−
17	..	..	..	++++	−	−	+++±	±	−
21	++++	−	−	..	..	..	..	..	..
24	..	..	..	++	−	−	+++	−	−

Tests with Chickenpox Vesicle Fluid and Chickenpox and Zoster Sera

Sufficient vesicle fluid was never obtained from a single case of chickenpox to permit the testing of a large number of sera. Consequently, individual fluids stored at − 70° were pooled for use. Most of the tests were made with 2 pools. In preliminary titrations one gave satisfactory serum complement fixation in a dilution of 1–13 and the other in a dilution of 1–28; as neither was anticomplementary they were used at these dilutions.

Table IV shows some of the results obtained with these antigens in titration of zoster and chickenpox sera. The results of previous tests of the same sera with zoster vesicle fluid ZPA. are shown for comparison.

It may be noted that with the chickenpox vesicle fluids the zoster sera again show higher titres than do those from chickenpox cases. Moreover, the titres given by individual sera are of the same order with both vesicle fluid antigens. There is nothing in these results to indicate that there is any qualitative difference in the complement-fixing antigens present in vesicle fluid from zoster or chickenpox patients.

Tissue Culture Fluids as Antigen

Tissue culture fluids which had been collected and stored as already indicated were tested for serological reactivity using a 1 in 8 dilution of convalescent zoster serum P.M. (Tables II and IV). As preliminary tests had shown that some failed to fix complement in the presence of potent convalescent serum they were only tested undiluted and in a dilution of 1 in 2. The results with selected fluids are shown in Table V. The 2nd column shows the length of time that the fluids had been in the tissue cultures at 37°.

It will be seen that the antigenic activity of the culture fluids was low. None gave a positive reaction when diluted 2-fold with saline and some failed to react even when undiluted. The antigenic activity of the fluid was not dependent only on the time of contact with infected tissue cells. Probably more important were the degree of infection of the cell sheet and the volume of the medium. Even in the presence of many infected cells fluid removed early may not be antigenic (ZE.) while if only a few infected cells are present then fluid may be inactive even after 36 (CJ.) or 43 days (CB. and CC.). The specific complement-fixing activity of tissue culture fluids devoid of horse serum can be increased by concentration

TABLE IV.—*Titration of Sera Using Pooled Chickenpox Vesicle Fluids and a Comparison of the Results with those Obtained with the Zoster Vesicle Fluid* ZPA.

Patient	Duration of rash when serum taken (days)	Zoster sera: Pooled chickenpox vesicle fluids (serum dilutions) 1/4	1/8	1/16	1/32	Zoster sera: Zoster vesicle fluid ZPA (serum dilutions) 1/4	1/8	1/16	1/32
A. B—	9	++++	++++	+++±	+	++++	++++	+++	+
	34	++++	+++	+	−	++++	++±	±	−
P. M—	4	+++	++	±	−	+++±	±	−	−
	26	++++	++++	++++	+++	++++	++++	+++±	++
J. N—	5	∓	−	−	−	∓	−	−	−
	16	++++	++++	+++±	++	++++	++++	+++	±
J. P—	5	++++	++++	++++	0	++++	++++	+++	++
	26	++++	++++	+++±	++	++++	+++	++	+
C. H—	3	∓	−	−	−	±	±	−	−
	25	++++	++++	+++±	+	++++	++++	+++±	+
I. W—	25	++++	++++	+++	+	++++	++++	++	±
S. A—	25	++++	+	−	−	++++	++	∓	−
K. L—$_2$	23	++++	++++	++++	++±	++++	++++	+++++	++
C. B—	23	++++	++++	+++±	+	++++	++++	+	±
A. T—	27	++++	++++	+++	±	++++	++++	+++±	+
S. F—	24	++++	++++	++±	+	++++	+++±	+	±
M. M—	26	++++	+++	+	−	+++±	++	±	−
Chickenpox sera									
M. A—	3	−	−	..	..	−	−	..	..
	19	+++±	+++	0	..	++++	++	∓	..
M. G—	4	−	−	..	..	−	−	..	..
	18	++++	0	..	..	++++	+	∓	..
P. D—	4	−	−	−	..	−	−	−	..
	25	++++	+++±	±	..	++++	+++	±	..
A. P—	3	∓	−	..	..	−	−	..	..
	24	+++±	++	±	..	++++	+	±	..
S. U—	3	−	−	..	..	−	−	..	..
	24	++++	++	−	..	+++±	+	−	..
P. O—	24	+±	−	−	−	+	−	−	−
I. T—	23	∓	−	−	−	−	−	−	−
G. S—	31	∓	−	−	−	±	−	−	−
H. I—	23	±	−	−	−	±	−	−	−
A. C—	24	+++±	+±	−	−	++++	++	±	−
D. T—	30	+	−	−	−	+	−	−	−
D. A—	18	++	−	−	−	++	−	−	−

0 = not tested.

using ultrafiltration (Weller and Witton, 1958) or possibly by freeze drying and reconstitution. However, our fluids contained 10 per cent horse serum and it was considered that concentration might increase their anticomplementary properties. Pooled positive fluids were therefore used undiluted in the tests of sera shown in Table VI.

Here again the results show no indication of differences in the complement-fixing antigens in tissue cultures infected with virus from zoster and chickenpox patients. The titres of the convalescent zoster sera, except for the 2 taken after 120 and 107 days, are again higher than those from chickenpox patients. With the chickenpox sera the titres are lower than those obtained with vesicle fluid antigens (Table IV). This is probably due to the low antigenic content of the

TABLE V.—*Tests of Tissue Culture Fluids for Complement-fixing Activity Using a $\frac{1}{8}$ Dilution of Convalescent Zoster Serum*

Culture fluid	Duration of contact with infected cells (days)	Degree of infection in cultures*	Zoster tissue culture fluids: Dilutions: Undiluted	Zoster tissue culture fluids: Dilutions: 1/2	Zoster tissue culture fluids: Antigen controls, undiluted
ZA.	5	+++	−	−	−
ZB.	9	+++	++++	∓	±
ZC.	9	+	−	−	−
ZD.	13	++++	++++	−	++±
ZE.	8	++++	−	−	+
ZF.	6	++	−	−	−
ZG.	12	++++	+++	−	−
ZH.	27	++++	++++	±	−
			Chickenpox tissue culture fluids		
CA.	32	+++	++++	−	±
CB.	43	+	−	−	−
CC.	43	+	−	−	−
CD.	43	++++	++++	−	−
CE.	13	++++	++++	∓	∓
CF.	40	+++	+++±	−	−
CG.	20	+	−	−	−
CH.	52	++	++++	+	−
CI.	36	±	−	−	−

Serum controls negative.
* Increasing degrees of infection indicated by + to ++++.

tissue culture fluids. It has been our experience that the use of a poor antigen reduces the titre of a weak serum more than that of a strong serum.

The results of tests on those sera, collected between the 5th and 40th days, which were examined in parallel against both chickenpox and zoster antigen are summarised in Table VII. A + + reaction was taken as the end point. It will be seen that nearly half the chickenpox sera failed to fix complement in a dilution of 1–4 and none were positive in a dilution higher than 1–8 with either antigen. None of the zoster sera were negative at 1–4 and nearly half had a titre above 1–8.

The Failure to Demonstrate Complement-fixing Antibody in Sera from Persons with a Past History of Chickenpox

It appeared from our earlier tests that in chickenpox complement-fixing antibody reached its peak in the 2nd or 3rd week after onset of illness and disappeared after about 3 or 4 months. To confirm this latter point sera were collected from 40 individuals who had had chickenpox between 5 and 50 years previously. Of these, one had also had zoster 4 years, and another 10 years, previously.

All sera were tested in dilutions of 1/2, 1/4 and 1/8 against a zoster vesicle fluid. None of the 40 sera gave a positive reaction even in a dilution of 1 in 2. However, 6 of these sera, when tested later, were found to contain neutralizing antibody. This is in accordance with the finding in other infections that neutralizing antibody can often be demonstrated long after complement-fixing antibody has ceased to be detectable.

TABLE VI.—*Titration of Zoster and Chickenpox Sera Using Chickenpox and Zoster Pooled Tissue Culture Fluids*

Patient	Duration of rash when serum taken (days)	Zoster sera: Pooled chickenpox tissue culture fluids (serum dilutions) 1/4	1/8	1/16	1/32	1/64	Zoster sera: Pooled zoster tissue culture fluids (serum dilutions) 1/4	1/8	1/16	1/32	1/64
J. P—	5	++++	++++	++++	+++	++	++++	++++	++++	+++	++
	26	++++	++++	++++	+++	−	++++	++++	++++	++	++
M. H—	2	−	−	−	−	−	−	−	−	−	−
	120	−	−	−	−	−	−	−	−	−	−
A. L—	3	−	−	−	−	−	−	−	−	−	−
	107	−	−	−	−	−	−	−	−	−	−
E. H—	4	−	−	−	−	−	−	−	−	−	−
	32	++++	++++	++++±	+	−	++++	++++	++++±	++	−
R. D—	5	±	−	−	−	−	±	−	−	−	−
	35	++++	++++	++++	+++	+	++++	++++	++++	++	+
L. A—	2	−	−	−	−	−	−	−	−	−	−
	22	++++	+	±	±	−	++++	++±	±	∓	−
E. F—	5	−	−	−	−	−	−	−	−	−	−
	31	+++	±	−	−	−	+++	+	−	−	−
Chickenpox sera											
M. G—	4	−	−	−	−	−	−	−	−	−	−
	18	+	−	−	−	−	+	−	−	−	−
A. P—	3	−	−	−	−	−	−	−	−	−	−
	24	++	−	−	−	−	+	−	−	−	−
P. D—	4	−	−	−	−	−	−	−	−	−	−
	25	++	∓	−	−	−	++	∓	−	−	−
J. B—	3	−	−	−	−	−	−	−	−	−	−
	120	−	−	−	−	−	−	−	−	−	−
I. T—	2	−	−	−	−	−	−	−	−	−	−
	23	−	−	−	−	−	−	−	−	−	−

TABLE VII.—*A Comparison of Sera Collected from Zoster and Chickenpox Cases Between the 5th and 40th Day from the Onset of the Rash and Tested in Parallel Against Zoster and Chickenpox Antigens*

Titre of sera	Zoster sera		Chickenpox sera	
	Zoster antigen	Chickenpox antigen	Zoster antigen	Chickenpox antigen
<1/4	0	0	7	8
1/4	6	9	5	4
1/8	9	5	4	4
1/16	7	7	..	..
1/32	4	5	..	..
1/64	1	1	..	..
	27	27	16	16

The Soluble Nature of the Antigen in Zoster Vesicle Fluid

Four ml. of diluted zoster vesicle fluid (ZJO.) was centrifuged at 20,000 rpm (36,200 *g*) for 30 min. in a Spinco ultra-centrifuge. The clear supernatant fluid was removed to a level just above the pellet-like deposit, the remaining fluid being discarded. The deposit was resuspended in fresh culture fluid to the same volume as the supernatant. Both the supernatant fluid and resuspended deposit were then titrated against a convalescent zoster serum (taken 24 days after the onset of the rash). The dilutions shown in Table VIII are in terms of the original volume of vesicle fluid.

TABLE VIII.—*Titration of* ZJO. *Supernatant Fluid and Resuspended Deposit with Convalescent Zoster Serum*

	Convalescent zoster serum (Serum dilutions)					Antigen controls (Doses of complement)		
	1/8	1/16	1/32	1/64	1/128	2	1½	1
ZJO (supernatant fluid dilutions)—								
1/20	++++	++++	+++	+	−	−	−	−
1/40	++++	+++	±	−	−	−	−	−
1/80	++++	+	−	−	−	..	..	..
1/160	±	−	−	−	−	..	..	..
ZJO (resuspended deposit dilutions)—								
1/20	++++	++++	++++	−	−	−		±
1/40	++++	++	±	−	−	−	−	∓
1/80	±	−	−	−	−	..	..	..
1/160	−	−	−	−	−	..	..	..
Serum controls (doses of complement)—								
2	−	−	−	−	..	..	..	..
1½	−	−	−	−	..	..	..	..
1	+	∓	∓	−	..	..	..	..

The speed of centrifugation was well above that necessary to deposit zoster virus particles (the No. 40 Spinco rotor deposits particles of 100 mμ diameter in 22·5 min. at 20,000 rpm (36,200 *g*). In tissue culture, the resuspended deposit was found to be infective whilst the supernatant fluid was not. One must conclude,

therefore, that the marked complement-fixing power of the supernatant fluid was due to soluble antigen. The complement-fixing activity of the resuspended deposit, less than that of the supernatant fluid as indicated by the lower dilution reacting with the immune serum, was presumably due to antigen associated with the virus particles and cell debris in the deposit.

DISCUSSION

Vesicle fluids from zoster and chickenpox cases provided satisfactory antigens for complement-fixation tests with convalescent sera. Individual specimens of fluid varied in reactivity and occasional samples gave satisfactory results when diluted 1–40 or 1–50. Tissue culture fluids on the other hand were less reliable and only some specimens were useful and then only when undiluted. Both the extent of infection in the cultures and the duration of contact of the fluid with the infected cells seemed to be of importance in determining the antigenic content of the fluid. Fluids were inactive unless they had been in contact with infected cells for at least 7 days. Weller and Witton (1958) changed the culture medium (bovine amniotic fluid) every 3 or 5 days and had to concentrate the culture fluids by ultrafiltration before they were of use in complement-fixation tests.

Convalescent sera from chickenpox cases reacted equally well with chickenpox or zoster antigens, whether vesicle fluids or tissue culture fluids. Similarly there was no evidence of greater reactivity of zoster convalescent sera with zoster than with chickenpox antigens. These results indicate, therefore, that the complement-fixing antigens of the viruses of chickenpox and zoster are identical. This conclusion is in accordance with the findings of Netter and Urbain (1926), Brain (1933) and Weller and Witton (1958) and is at variance with the results of Hasskó *et al.* (1938).

The results on sera taken early in the disease reckoned from the onset of the rash suggest that antibody appears earlier in zoster than in chickenpox cases. This was also noted in some of Weller and Witton's sera and it was suggested by them that while this finding might be a reflection (in zoster cases) of previous experience with varicella virus, it might also reflect the existence of a longer prodromal period in zoster cases. In our tests, however, the titres of complement-fixing antibody in convalescent sera were generally higher in zoster than in chickenpox. There is no reason to believe that the more localized infection in zoster should of itself call forth a higher antibody response than the generalized infection of chickenpox. Our findings are compatible with the view that many, if not all, cases of zoster have had previous infection with varicella virus, and that the higher antibody titres observed indicate a secondary response to infection with the virus. Weller and Witton on the other hand did not record any marked difference in titre of complement-fixing antibody in the sera of chickenpox and zoster cases. Of their sera collected from chickenpox cases between the tenth and thirtieth days after the appearance of the rash, all gave positive results, whereas more than half of ours were negative at this time. Moreover, they recorded positive reactions persisting longer after the acute illness than were obtained in our series. This, presumably, reflects a difference in sensitivity of the techniques used, and the concentrated tissue culture antigen used in their tests may have been in part responsible for this. The apparently less sensitive method used by us may have served to bring out the quantitative difference in the antibody content of sera

from chickenpox and zoster cases. A similar difference was observed by precipitation tests and will be recorded in a later paper.

SUMMARY

Sixty-six sera from 35 cases of zoster and 81 sera from 25 cases of chickenpox were tested in serial dilutions for complement-fixing antibody with zoster vesicle fluid. Convalescent zoster sera showed higher titres than did convalescent chickenpox sera. Although smaller numbers of sera were tested against chickenpox vesicle fluid the zoster sera again gave higher titres. The tests on the same sera with the 2 types of antigen showed remarkably close correlation, suggesting that the complement-fixing antigens of the viruses are identical. By high speed centrifugation of diluted vesicle fluid the complement-fixing antigen could be separated from infective virus and still retain its reactivity. Tissue culture fluids had weak antigenic activity compared with vesicle fluid. Despite this, the results with convalescent zoster and chickenpox sera gave no indication of an antigenic difference between zoster and chickenpox culture fluids.

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THE PROPERTIES OF CRYSTALLINE DIPHTHERIA TOXIN-PROTEIN: THE REACTION OF THE PHOSPHATE AND PEPSIN-STABLE ANTIGENS WITH DIPHTHERIA ANTITOXIC SERUM

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A crystalline material isolated from culture filtrates of *Corynebacterium diphtheriae*, with both the *in vivo* and *in vitro* properties of the specific toxin (Pope and Stevens 1953*a*, 1958*a*), has been shown to contain a number of distinct antigens (Pope 1957, Pope and Stevens 1958, *b*, *c*, *d*). One of these, present in all batches of crystalline toxin-protein available at that time (Pope 1957) had the properties of a deoxyribonuclease. Subsequent work has shown that this antigen is present in crude culture filtrates obtained by growing *C. diphtheriae* on tryptic digest medium but is absent from those grown on papain digest medium. Consequently its presence is restricted to crystalline toxin-protein isolated from tryptic digest culture filtrates; thus it would not appear to be connected with the specific toxin. The presence in several batches of commercial antitoxin of an antibody capable of inhibiting the activity of this enzyme, and the recovery of the enzyme activity from the washed toxin-antitoxin floccules, is accounted for by the fact that the horses had been immunized with toxoid prepared from tryptic digest culture filtrates. Similarly, another antigen that is stable to trypsin has been shown by Pope and Stevens (1958*d*) to be frequently, but not invariably, present in crystalline toxin-protein.

Of the antigens which always appear to be present, even in material crystallized 10 times, 2 have been isolated in a reasonably pure immunological state and it has been possible to study the interaction of each with its own antibody and to show that neither reacts with the specific-antitoxin. This is a matter of some importance in its bearing on the composition of the toxin-antitoxin floccules.

MATERIALS AND METHODS

These have been described previously (Pope and Stevens 1958*c*).

Crystalline toxin-protein showing 4 diffusion lines

For use in the Ouchterlony gel-diffusion plate test it was convenient to have available a modified toxin that showed 4 well-defined diffusion lines. Material for this purpose was prepared in the following way. Crystalline toxin-protein solution (1000 Lf/ml.) to which had been added 0·1 per cent anhydrous Na_2SO_3 was incubated at 37° (pH 8·5–9·0) for 48 hr. Gel-diffusion tests showed 4 well-defined lines. The preparation was dialysed to remove any excess sulphite. Flocculation tests showed 700 units/ml., and intracutaneous tests 500 units/ml. Sodium sulphite, used as described, gives a well-defined separation of the diffusion lines produced by the 4 antigens with a relatively small change in the specific toxicity. This material is of value in diffusion tests for identifying the different antigens and following the course of their destruction.

Fig. 1 illustrates the diffusion lines shown by the original crystalline toxin-protein and the modified toxin prepared from it, tested against pepsin-refined antitoxin. The lines numbered 1 and 2 are due to antigens both of which are relatively stable to pepsin at pH 3·7 although, in general, the antigen responsible for line 1 is rather more susceptible to this treatment. Line 3 is produced by the phosphate-stable antigen and line 4 is due to an antigen not yet isolated and identified.

EXPERIMENTAL

Preparation of the pepsin-stable antigen

Crystalline diphtheria toxin-protein solution (2600 Lf units/ml.) was diluted with 2 volumes of 0·05 M phosphate buffer (pH 3·4) and the mixture was adjusted to pH 4·0. Crystalline pepsin, dissolved in the minimal amount of 0·01N HCl,

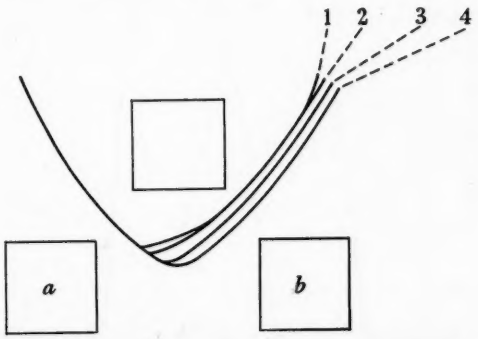

FIG. 1.—The diffusion lines produced by (*a*) the crystalline toxin protein, (*b*) the same toxin after sulphite treatment.

Lines 1 and 2 are due to the antigens relatively stable to pepsin at pH 3·7. With some batches of crystalline toxin-protein it is difficult to get good separation of these two lines. Line 3 is produced by the phosphate-stable antigen and line 4 is produced by an antigen not yet isolated and identified. The top reservoir contained pepsin-refined antitoxin.

was added (5·0 mg. per 100 ml. of diluted toxin) and the pH finally adjusted to 3·7. After standing for 3 hr. at 18° the pH was adjusted to 8·0 with 2N NaOH. The time allowed for digestion at pH 3·7 and 18° should be determined for each batch of crystalline toxin-protein. After removal of a very small precipitate, the apparent Lf value was found to be 500 units per ml. and the yield was approximately 60 per cent. The digested material was fractionated by adding 20 per cent w/v $(NH_4)_2SO_4$ and removing a small precipitate, after which, the remaining antigen was precipitated at a final concentration of 50 per cent w/v $(NH_4)_2SO_4$. The precipitate was dissolved in 0·1 per cent w/v $NaHCO_3$ solution and dialysed. About 65 per cent of the apparent Lf units present in the digested toxin were recovered. Flocculation tests against a pepsin-refined antitoxin gave a value of 744 u/ml. by the β method and 693 u/ml. by the α method. When examined by total combining-power methods in the presence of excess antitoxin, *in vivo* tests showed that 1·0 ml. of this product neutralized 12 units of specific antitoxin. Comparing the high apparent Lf value and the specific combining value it is seen that the pepsin-stable antigen is almost free of specific toxin.

The reaction of the pepsin-stable antigen with pepsin-refined antitoxin was studied by the method used in earlier work (Pope and Stevens 1958*c*). As this antigen forms but part of the total antigenic system present in crystalline toxin-protein the amount of antitoxin was held constant at 60 u/ml. in the final mixtures (3 times the usual amount) in order to get a good floccule precipitate. The α value of 693 u/ml. was used as the basis for calculating the amounts of antigen required. Results from this experiment are shown in Fig. 2, from which it will be seen that

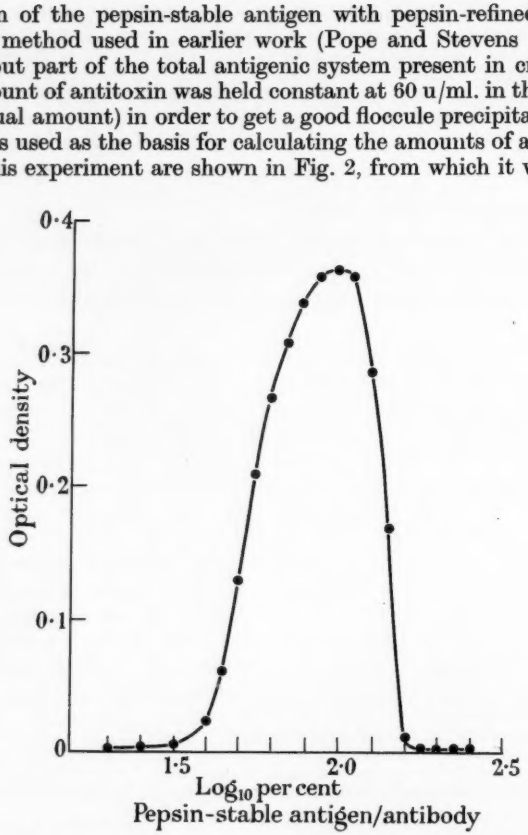

FIG. 2.—The flocculation reaction between the pepsin-stable antigen and pepsin-refined antitoxin. All mixtures had a constant volume of 10·0 ml. and each contained 600 units of antitoxin.

the shape of the curve is quite smooth with its peak corresponding to the optimal flocculating mixture. This curve differs very markedly in shape from those obtained by the interaction of crystalline diphtheria toxin-protein with pepsin-refined antitoxin (Pope 1954, Pope and Stevens 1958*c*, *d*) and resembles more closely those from pepsin-refined antitoxin from which many non-antitoxic antibodies had been removed by absorption (Pope and Stevens 1953*b*, *c*).

The possibility that this preparation of pepsin-stable antigen may contain some of the material responsible for line 1 shown in Fig. 1 must be considered. Its presence could account for the fact that the curve, shown in Fig. 2, is less symmetrical than that obtained from the phosphate-stable antigen (See Fig. 3).

The abscissae for Fig. 2 are given in the form $\log_{10}$ per cent pepsin-stable-antigen/antitoxin because it was considered possible that some other antigenic residue

might be found when large amounts of the pepsin-stable antigen was used. However there was no sign of any other reacting system above the $\log_{10}$ value of 2·4.

Free specific-antitoxin present in the total mixtures, and in the supernatants from those mixtures which contained floccule precipitate, was estimated by means of intracutaneous tests in guinea-pigs. No significant difference was found between the antitoxin values of the total mixtures or supernatant. In all mixtures across the range 20 to 200 per cent antigen/antitoxin the amount of free specific-antitoxin found was 80 per cent or more of that theoretically possible. This is in striking contrast to the behaviour of the crystalline toxin-protein in its reaction with

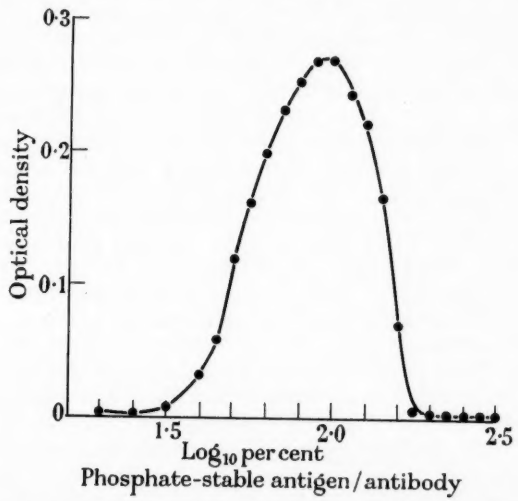

FIG. 3.—The flocculation reaction between the phosphate-stable antigen and pepsin-refined antitoxin. All mixtures had a constant volume of 10·0 ml. and each contained 600 units of antitoxin.

antitoxin where, over the range 50 to 160 per cent toxin/antitoxin, virtually 100 per cent of the specific antitoxin is present in the floccule precipitate. The small loss of specific antitoxin found in the reaction between the pepsin-stable antigen and antitoxin was due to the trace of combining material, already mentioned, left in this preparation, and not to the major reaction between the pepsin-stable antigen and its own antibody.

Preparation of the phosphate-stable antigen

The action of 5 per cent K_3PO_4 on crystalline toxin-protein has been described by Pope and Stevens (1958*c*). An apparent doubling of the Lf value was shown to be due to the presence of an antigen, stable to the treatment used, that reacted with its antibody to give a smooth, almost symmetrical curve. The optimal flocculation point, and the peak of the curve were coincident in a mixture of the composition 55 per cent phosphate-toxin/antitoxin; this accounted for the apparent increase in Lf value. It was also shown that other antigens present

in the original toxin became non-flocculating without a complete loss of power to combine with their antibodies. This product was not, therefore, suitable for use where it was desired to study the relation between this antigen/antibody system and the specific antitoxin.

An alternative method using Na_2SO_3 was found which led to the isolation of the desired antigen, with a much greater destruction of the other antigens present in crystalline toxin-protein, and a much lower combining power with specific antitoxin as measured by *in vivo* tests. The apparent increase in Lf value was similar to that obtained with K_3PO_4, and diffusion tests by the Ouchterlony method showed a reaction of identity between the single line given by the product and that of material prepared by the phosphate method. Other antigens present in crystalline toxin-protein do not show a similar reaction of identity by this test. Since the antigen obtained by the new method appears to be the same as that obtained by means of K_3PO_4, the term " phosphate-stable antigen " has been retained to avoid confusion. The new method depends on the action of Na_2SO_3 at pH 11 and 35° on the crystalline toxin-protein : either NaOH or preferably solid K_3PO_4 may be used to adjust the pH value. For the experiment reported, the antigen was prepared in the following way : to crystalline toxin-protein solution (700 Lf units/ml.) was added 0·5 per cent w/v anhydrous Na_2SO_3 and and 0·25 per cent K_3PO_4. After heating at 35° samples were removed at intervals for flocculation tests. Direct tests showed the expected increase in apparent Lf value from 700 Lf units/ml. to a new value of 1400 units, and this value then remained constant. In order to follow the other antigens, which were undergoing destruction, blend tests were carried out. The value obtained in this way decreased steadily until, after 9 days at 35°, it was 12 units per ml. The real meaning of this test is not clear; it is not known what it measures, but experience has shown that when the value found by this test has fallen to less than 2 per cent of the original Lf value, little combining power is left relative to the specific antitoxin.

The product was dialysed against distilled water to remove excess sulphite and phosphate. In order to remove the last traces of antigen capable of combining with the specific antitoxin an amount of pepsin-refined antitoxin calculated on the basis of the low blend value was added, followed by $(NH_4)_2SO_4$ 20 per cent, to precipitate the antigen-antibody complex formed. After filtration, the proteins present in the filtrate were precipitated by increasing the $(NH_4)_2SO_4$ to 45 per cent, separated and dialysed. Tests on the final product showed that the blend value was now zero units per ml., while the direct flocculation test (α method) showed an apparent value of 817 Lf units per ml.

This method has been found to be of value for removing traces of unwanted antigens provided there is a large difference between the value of the main antigen and that of the residual traces of other systems.

The reaction between this antigen and its antibody was determined by the optical method exactly as described for the pepsin-stable antigen. This phosphate-stable antigen/antibody reaction produced a smooth and narrow curve, Fig. 3, similar to, but more symmetrical than that shown in Fig. 2. For a constant amount of the pepsin-refined antitoxin, the phosphate-stable antigen produced, by comparison with the pepsin-stable antigen, slightly less precipitate at the peak of the curve. All the total mixtures, and the supernatants from mixtures containing floccules, were tested for the presence of free specific-antitoxin (*in vivo* tests). In every case more than 80 per cent of the antitoxin theoretically possible was

found to be present. The phosphate-stable antigen does not neutralize the specific antitoxin except for trace amounts which could be accounted for if a little specific toxin-antigen remained in this antigen preparation.

Quantitative Aspects of these Antigen/Antibody Systems

In previous work (Pope and Stevens 1958*c*, *d*) it has been shown that with the refined peptic-antitoxin used, the phosphate-stable antigen reacts optimally at 55 per cent toxin to antitoxin. Similarly, the pepsin-stable antigen or antigens react optimally at 80 per cent toxin to antitoxin. These values relate to the original curve given by the crystalline toxin-protein with this antitoxin, where the optimal flocculation point (α procedure) is taken as 100 per cent. Thus both these antigens react mainly in the excess antitoxin region. Since the amount of pepsin-refined antitoxin in all mixtures used in the optical method was constant, the amount of the different antibodies was also constant.

Using the data obtained from the present experiments, calculations were made and the curves redrawn on the basis of 20 units of antitoxin per ml. for each mixture (as in the original toxin/antitoxin curve). Each curve was adjusted to the point corresponding to the peak of the antigen involved *i.e.* 55 per cent for the phosphate-stable and 80 per cent for the pepsin-stable antigens. When these 2 curves were added together the resultant curve showed maximal precipitation at 70 per cent toxin/antitoxin and the sum of these 2 antigen precipitates accounted for about 65 per cent of the total precipitate given by the crystalline toxin-protein in this area of its curve. This should be regarded only as a preliminary attempt to assess the contribution made by each of the antigens present in the crystalline material ; at present only 2 have been isolated. Even now this figure of 65 per cent does not take account of the co-precipitation of polymerised antigen-antibody which would take place were both antigens present at the same time, neither does it take into account the polymers from other antigen-antibody systems that are also partially neutralized, which may be co-precipitated. Nevertheless, this result indicates that these 2 antigens are major, rather than minor, contributors to the Ramon floccule precipitate produced by the reaction of crystalline toxin-protein with pepsin-refined antitoxin.

DISCUSSION

The isolation of 2 antigens from crystalline diphtheria toxin-protein in reasonably pure immunological state has allowed a study of their behaviour with pepsin-refined antitoxin. Each antigen has been shown to produce a narrow, smooth, and approximately symmetrical flocculation curve with its own antibody. In each case it was found that more than 80 per cent of the specific antitoxin theoretically present was unneutralized, or precipitated in the floccules. Very small losses of specific antitoxin could be accounted for in terms of the trace of combining power associated with these isolated antigens. Moreover, these isolated antigens have the property of reacting with peptic antitoxin ; they flocculate with their own specific antibody to yield supernatants that give no line in the gel-diffusion test with the antigen used, but retain the ability to give lines with the other antigens present in the crystalline toxin-protein. Absorption with the phosphate-stable antigen may be followed by absorption with the pepsin-stable antigen to give a supernatant that no longer reacts to either antigen ; but it still gives a diffusion

line with another antigen (Fig. 1 line 4) present in the original toxin-protein. The evidence for the existence of these specific antibodies as entities in refined peptic-antitoxin appears to be clear and supports the results of Pope and Stevens (1958*b*) who showed variation of these antibodies in the serum of individual immunized horses.

The presence of a series of distinct antigens in crystalline diphtheria toxin-protein was disclosed as a result of the application of methods such as heat denaturation, chemical treatment, or enzyme action. It is difficult to believe that such varied forms of treatment would lead to the breakdown of a complex molecule to yield a recognizable pattern of antigens with specific antibodies in horses immunized with untreated toxin (or toxoid). Evidence that these antigens exist as entities in the crystalline toxin-protein is still needed, and work on this is in progress.

Some preliminary evidence is available from the results obtained from the interaction of 2 of the antigen systems with their antibodies to show the manner in which these 2 systems fit into the flocculation curve for the original crystalline toxin. In the region slightly on the excess antitoxin side of the neutral point the sum of these 2 antigen/antibody systems accounts for 65 per cent of the Ramon floccule precipitate given by the original toxin. If these 2 systems were present together, then as a result of co-precipitation of antigen/antibody polymers, the total precipitate would be more than 65 per cent. In addition, the other antigens present in crystalline toxin-protein would also be partially neutralized at this point, and these polymers would also be co-precipitated. This evidence makes it quite clear that the Ramon floccule precipitate cannot be accounted for in terms of a specific-toxin/specific-antitoxin precipitate, since much of the precipitate is produced by other antigen-antibody systems.

SUMMARY

The phosphate-stable and pepsin-stable antigens have been isolated from crystalline diphtheria toxin protein in reasonably immunologically-pure form to allow a study of their reaction with their own antibodies present in pepsin-refined antitoxin. They give antigen-antibody precipitates whose curves are narrow, smooth and almost symmetrical in shape.

These antigens do not neutralize the specific antitoxin nor is it carried down in the floccule precipitates. At the point of optimal precipitation more than 80 per cent of the specific antitoxin is free in the supernatant.

A preliminary attempt has been made to determine the contribution of these 2 antigen/antibody systems to the Ramon floccule precipitate produced when crystalline diphtheria toxin-protein and pepsin-refined antitoxin are used. The results obtained indicate that they contribute at least 65 per cent.

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SURVEY OF PAPERS

NOSSAL prepared cell suspensions from the popliteal lymph nodes of rats which had been injected in the hind foot pads with a flagellar antigen prepared from *Salm. adelaide*. These cells were isolated in micro-droplets and motile *Salm. adelaide* put into such a droplet were immobilized if the cell therein was producing antibody. Later, fixative and stain were instilled into the droplets enabling the histological nature of the cell to be confirmed. Of 601 cells thus classified, 93 made antibody and 91 of these were plasmablasts or plasma cells (p. 301).

ZEMPLÉNYI and GRAFNETTER produced atherosclerosis in male rabbits by cholesterol feeding and assessed the amount of lipolytic activity in the heart and aorta. The experiments are compared with those of a previous investigation performed on rats and they found that after prolonged cholesterol feeding in rabbits the lipolytic activity of myocardial tissue was greatly increased. This finding was less marked in aortic tissue (p. 312).

NERURKAR and SAHASRABUDHE have confirmed that the level of liver glycogen in fasting rats is raised by X-ray irradiation. The increase affects both " bound " and " free " glycogen and appears to result from increased glycogenesis rather than glyconeogenesis (p. 318).

ALLISON and MARGIE LANCASTER investigate the changes in endothelial permeability in the ears of rabbits after injury by heat and subsequent *in vivo* intravenous administration of Evan's Blue Dye. Using the ear-chamber technique they found that heat injury produced an increased vascular permeability which persisted for up to 24 hr., and that this leakage was most copious in vessels at points near arterial communications. It was also noted that stasis of blood flow occurred in association with this increased vascular permeability although the phenomenon could not be causally related (p. 324).

CAMPBELL, CHURCH, DICK, LAWRIE, PIRRIE and WATSON fed Golden Legbar-Light Sussex cockerels on a cholesterol diet to produce atherosclerosis in order to study the anti-atherogenic properties of the synthetic oestrogenic substance epi-oestradiol. The authors find that even with large doses of this substance no improvement in the coronary or aortic atheroma resulted (p. 338).

LEVINE prepared quantitative flocculation curves for reactions between 21 different pairs of partially purified tetanus toxoids and antitoxins. He found that the main tendency was to form linear zones within a limited range of combining proportions. The interrelationships of 4 definite parameters of these zones, namely slope, intercept and coordinates of the beginning and end of the zone were investigated. The slope was found to be correlated with the specific activities of the toxoids and the intercepts with the specific activities of the antitoxins. It was postulated that if the slopes and intercepts are measures of immunochemical purity of toxoids and antitoxins then the coordinates of the extremities of the zone lines would be linear functions of the ratio of intercept to slope for any reacting pair. Such linear functions were found and they provided the coordinates for constructing theoretical zone-lines for reactants of any assumed values of purity. The experimental zones fitted the theoretical zones in a manner unlikely to be due to chance. He concluded that the zone lines represented true regions of equivalence in which essentially complete precipitation of both reactants had occurred (p. 343).

WHITBY and ROWLEY cultured mouse peritoneal macrophages after these had been infected *in vivo* with various bacteria. They showed that organisms pre-treated with specific antibody were rapidly ingested and destroyed *in vivo* and *in vitro*. Untreated

bacteria were only destroyed by macrophages when phagocytosed, elimination being more rapid from immune than from normal mice (p. 358).

BERG, using a strain of Mendel-Sherman rats, found a high incidence of duodenitis and duodenal ulcers when the animals were fed on a pantothenic acid deficient diet. Duodenitis was produced after 8–12 weeks of this deficiency diet in 90 per cent of the animals with ulceration in 60 per cent. The experiments show that pantothenic acid is necessary for the maintenance of an intact duodenal mucosa and that duodenitis is a precursor of ulcer formation. Although in humans there is little likelihood of spontaneous pantothenic acid deficiency it is possible that interference with its metabolism might be a factor in the production of human duodenal ulceration (p. 371).

CORSI, GALLUCCI and PAOLA BARGELLINI have found abnormalities in the structure of myofibrils from vitamin E deficient rabbits. Although the amount of myosin which could be extracted from the myofibrils of deficient rabbits was normal, it was bound to a greater extent with actin (p. 375).

MACKANESS has investigated the effect of pregnancy upon the renal response to renin in the rat. Since pregnant animals show little rise in blood pressure when injected with renin, he studied the diuretic and natriuretic responses. He found that the diuretic effect of renin was diminished in pregnant animals as was the natriuretic response. In both cases the magnitude of the response fell progressively as pregnancy advanced. High doses of renin were antidiuretic and this effect was also depressed in pregnancy. He concludes that these results are due to involvement of the renin-hypertensin reaction (p. 382).

LIKAR, BARTLEY and WILSON treated monkey kidney monolayers with crude filtrates of cultures of faeces from 3 individuals. The subsequent multiplication of type 1 poliovirus in the cells was diminished by one of the filtrates. Endotoxins of some of the organisms isolated from the same individual's faeces had the same effect ; so did, in greater degree, the glucolipid polysaccharide endotoxin of *Salm. typhimurium* (p. 391).

TAYLOR-ROBINSON and DOWNIE used herpes zoster and varicella vesicle fluids and fluids of tissue cultures infected with these viruses as antigens in complement fixation tests with sera from zoster and varicella patients. Results suggested that the complement fixing antigens of the two viruses were identical, and that most cases of zoster have been previously infected with varicella virus (p. 398).

POPE and MURIEL STEVENS have continued their investigation of the properties of crystalline diphtheria toxin-protein. They prepared the phosphate stable and pepsin-stable antigens sufficiently pure to study the reaction of these antigens with their own antibodies present in pepsin refined serum. These systems yielded flocculation reactions in which the curves were narrow, smooth and almost symmetrical. The specific diphtheria antitoxin was not affected by either antigen. A preliminary attempt has been made to determine the extent to which these two antigen-antibody systems contribute to the Ramon floccule precipitate between crystalline diphtheria toxin-protein and pepsin refined toxin (p. 410).

ANNOUNCEMENT

It is proposed to form a British Association for Cancer Research in order to bring together cancer research workers in this country and to facilitate the interchange of ideas in this field. With the approval of the British National Cancer Committee, arrangements have been made to hold a meeting in the University of Glasgow from 15th to 17th September, 1960. This meeting will be open to all members of cancer research organizations in Great Britain and Ireland, and it is hoped that there will be a representative attendance.

Further details will be available early in 1960.

J. G. Bennette (*Secretary*),
E. Boyland,
J. W. Jull,
P. R. Peacock.